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Neurology Volume 8 • Supplement 1

Introduction

Francis M. Forster, M.D.*

It is a privilege and honor to represent the American Academy of Neurology officially on this occasion, and welcome the Section on Neurochemistry into the fold of the Academy. The American Academy of Neurology and modern neurochemistry are essentially contemporaries. This is the ninth annual meeting of the Academy, and its genesis actually goes back some five years prior to the first meeting, when Dr. A. B. Baker, the first president and the founder of the Academy, began his campaign among the young neurologists of that day to organize a new society.

The "elder statesmen" of this Academy were graduating from medical school at the time the first modern text on chemistry of the brain was written by Irvine Page. During our early days in neurology, the term neurochemistry was not in use. Very few, if any, of us were initiated into the traditions of Thudichum. Chemistry as it related to neurology was entirely clinical, dealt with the study of cerebrospinal fluid, and was restricted to measurements of total protein, sugar, chlorides, and the mysterious colloidal gold reaction. Some of us, under the banner of neurophysiology, crossed that nebulous boundary between basic sciences and found later that we had excursed slightly, if unwittingly, into neurochemistry by pathways such as the acetylcholine reactions. Gradually, as we became chairmen of departments of neurology, we realized more and more the need for research in the field of chemistry of the nervous system and added to our departmental staffs scientists interested in this area. In our own personal experience, we have encountered first the need and then the satisfaction of the fulfillment of the need. This is now translated into the same experience for the American Academy of Neurology.

The American Academy of Neurology has traditionally been a society of and for clinical neurology. Clinical neurology remains the central core of the academy and its membership is bound together by its interest in the practical applications of the various areas within neurology to the clinical problems of this most fascinating field of medicine. Neurology is really more than a specialty. Neurology is a discipline because it includes so much more than clinical neurology. Were neurologists concerned only with the diagnosis and treatment of patients, neurology would, in fact, be a subspecialty. However, neurology has traditionally encompassed neuroanatomy and neuropathology within its scope. When the field of neurophysiology evolved, this too became part of the total framework of neurology.

In its early days the Academy in its scientific programs and in its publications, through its own journal NEUROLOGY, placed interest and emphasis on clinical neurology. However, the membership soon felt and expressed a desire to hear more of experimental neurology and to have more research orientation in the program and publications. The total framework of the program for the Academy gradually changed so that purely scientific papers in the basic sciences began to appear and then

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Read at the Colloquium on Neurochemistry during the ninth annual meeting of the American Academy of Neurology, Boston, April 26, 1957.

became more and more prominent in the program. The journal NEUROLOGY published these and neurologists expressed their satisfaction and abiding interest.

The Academy has shown a continual growth and development which is close to phenomenal, and within the past two years the membership of this society has doubled. It soon became obvious that either the bulk of the membership would remain inarticulate or some change had to be made in programming.

As most of you know, I have long been active in the American Medical Association and have understood the section framework within the A.M.A., having served in the Section on Nervous and Mental Diseases as secretary, chairman, and now as delegate to the house of delegates. The section format in the A.M.A. seemed to me to be an ideal one which might be developed in the American Academy of Neurology.

Shortly after obtaining approval of the board of trustees and officers for the setting up of a section type of meeting and the proviso that sections might be appointed, I had the privilege of meeting with Dr. Donald B. Tower, Dr. Maynard Cohen, and Dr. Elizabeth Roboz and discussed with them the potentialities of a section in neurochemistry. I was then able to take their formal application for the formation of the section before the board of trustees and officers. In this way, not only was the section authorized in principle, but the policy was implemented and today we are raising the curtain on the first section of the American Academy of Neurology. Judging by the program and the enthusiasm engendered by this section, the officers and board of trustees, and particularly myself, are especially gratified, for there can be no doubt that the Section on Neurochemistry is a viable product and one destined for a long and distinguished career.

It is the sincere hope of the American Academy of Neurology that the Section on Neurochemistry, by its growth and development within the framework of the Academy, will continue to add strength and luster to the Academy as it has already done. We hope that our membership, with its broad representation and its particular clinical interest, will, by the impact of this new section, be kept abreast of developments in the field of neurochemistry and that each member will have his appetite whetted to learn more about the most rapidly developing and most fascinating of all the basic disciplines of neurology.

It is our sincere hope, on the other hand, that the section will prosper and profit by remaining in the Academy. The section should not only influence the Academy but, by eclectic selection, choose from the activities of the Academy and its scientific programs those portions which appeal to it so that the Section on Neurochemistry may likewise derive inspiration and strength and stimulus from us. We trust that this section will not long remain in the solitary state of being the only section. We expect that sections in neuropharmacology and neurophysiology may also be organized in the not too distant future.

There might be some concern as to how the Academy can function with so many activities ensuing in the course of the meeting. This should offer no concern to the membership in the Section of Neurochemistry nor to the membership of the Academy at large. The officers and board of trustees envisage that in the future Academy meetings will consist of three parts: one part, the courses which have been so successful for some five years; one part, the general meeting; and a third part, the sections. As we develop, the general meeting will probably hold one session or two and then the section meetings will follow, with a final session, a general meeting. The sections, as they multiply, will probably have joint meetings just as occur in the A.M.A., and at times a section or combination of sections will have a joint meeting with the general membership. Where we shall stand 15 or 20 years from now is most difficult to predict. Perhaps the Section on Neurochemistry will have split into subsections on enzyme chemistry, radioisotope chemistry, or some other fractions. There may well be five or six or seven different sections other than those dealing with chemistry by that time, but certainly it is impossible to foresee anything but growth, strength, and development for neurology or neurochemistry and for the total research and development in the field of the nervous system, stemming from the efforts that you and the Academy as a whole are making today.

Origins and development of neurochemistry

Donald B. Tower, M.D., Ph.D.

CHEMICAL INVESTIGATIONS of the nervous system have progressed to the point that they now form a definite subdivision of neurobiology which we designate neurochemistry. There are already departments or laboratories in several institutions devoted to neurochemistry. Books on the subject have begun to appear. A series of international colloquia on neurochemistry has been inaugurated. An international journal has started publication. Now the American Academy of Neurology has established as its first neurologic specialty section a section on neurochemistry. We may well inquire how this development has occurred and what are its implications for neurology and neurologic research in the future.

Neurochemistry is not easily defined. Those of us working directly in this field do not always agree on its scope or limits. The various facets which might be included cover a very wide range. To obtain a clearer understanding of the situation, it is worthwhile to look back over some 300 years to trace the origins and development of neurochemistry. In doing so, we should take a broad view of what constitutes neurochemistry and realize the close correlation of its development with that of neurophysiology and biochemistry.

It is not feasible here to follow these developments in great detail, nor has it been possible to consult all the original sources which a full historical treatment would require. Many of these sources are difficult to identify and locate, and such a study is hampered by a lack of adequate histories of the development of neurophysiology and of biochemistry. Most historical accounts are primarily biographic in form. It is undeniable that people make history and some figures dominate certain periods. For our purposes, however, emphasis on the development of ideas and concepts would seem to be more pertinent than biographic cataloguing. As Fearing[89] has pointed out, it is often futile or impossible to attempt to determine exactly who first discovered a phenomenon and when, since thought and development in science are not usually discontinuous processes, but observations may long precede the urge to record or demonstrate them.

Of necessity, considerable dependence has been placed on secondary sources. These are not always written with our present purposes in mind, and it is difficult to eliminate or to avoid interjecting personal viewpoints. It is not even certain that all of the important developments in our story have been recognized, particularly since we may not yet appreciate the full significance of some ourselves. There are, therefore, some limitations which must be borne in mind in considering the data to follow.

PRELUDE TO THE MODERN ERA

Historians of science are generally agreed that until the sixteenth century, the time of the Renaissance, European science was dominated by the findings and ideas handed down from Greek and Roman times. The reasons for this long period of fallowness in scientific thought and endeavor are sufficiently familiar to need no elaboration here. Medical thought was dominated by the teachings of Galen [129-199 A.D.], whose treatises are among the few which survived to come down to us relatively extant. Much of what we know depends upon the labors of this physician and investigator of imperial Rome in the time of Marcus Aurelius in recording the bulk of knowledge in the field of medicine at that time. These writings were accepted throughout the medieval period as unquestioned fact.

From the section of clinical neurochemistry, National Institutes of Neurological Diseases and Blindness, Bethesda, Maryland.

Presented in abbreviated form at the Colloquium on Neurochemistry during the ninth annual meeting of the American Academy of Neurology, Boston, April 26, 1957.

Chemistry as such did not exist in ancient times. There were the practical arts of fermentation (wines and vinegar), pottery making, crude metallurgy, glass making, and dyeing, for a knowledge of which we are indebted to the *Natural History* of Pliny the Elder [23-79 A.D.]. In addition, there was the rise of alchemy, first perhaps in Egypt but soon permeating all civilizations, a combination of mysticism, magic, and astrology which sought to transmute base metals into gold, the perfect medicine and source of eternal life, by means of the "essence" or philosopher's stone. The alchemists made use of the practical methods of their brother artisans and eventually laid a foundation for the rise of true chemistry as a by-product of their fruitless search.[45,94]

Knowledge of the nervous system in those days was remarkably good. Such knowledge was undoubtedly acquired by observations over a long period. Trephination and trepanation were practiced long before recorded history. Head injuries were among the subjects covered in the Edwin Smith Papyrus of 1600 B.C.[20] By the sixth century B.C. the Greeks recognized the brain as the center of higher activities [Alcmæon of Crotus; Pythagoras of Samos]. Clinical observations on cases of head injury, epilepsy, and the like led to early ideas of localization and contralateral innervation [Hippocrates: 460-370 B.C.]. Experimental studies demonstrated the effects of cord and nerve section and decapitation [Aristotle: 384-322 B.C.], the vulnerability of the hindbrain to fourth ventricle puncture [Herophilus: ca. 344 B.C.], and the connection of the nerves with the brain and their role in subserving sensation and motion [Erasistratus: 300-250 B.C.]. By the time of Galen in the second century A.D. the anatomy of the central nervous system was known in detail, the brain was recognized as the seat of intelligence, voluntary movement, and sensation, motor and sensory nerves were distinguished, and the effects of sections and crude ablations at various levels experimentally established. By this time, as Soury[112] has pointed out, the anatomy and much of the basic physiology of the nervous system was as well advanced as at the time of Willis and Vieussens 1,500 years later.

The precursors of physiologic chemistry were not as striking. In part this is due to the lack of adequate methods and knowledge, but it may also be due to a lack of surviving records. Poisons such as curare, ouabain, and veratrine and drugs such as opium, hashish, coca, cinchona, and mandragora (belladonna) were known to the ancients.[45] Opium and mandragora were used to ease pain and continued to be mainstays of the surgeon throughout the Middle Ages. The Assyrians knew that carotid compression would produce "analgesia" and the Greeks called these vessels *soporales*.[62] Mine gases and what we now know to be carbon monoxide were early recognized to be brain "poisons" [Erasistratus; Galen; Avicenna].[27] The term albumen was introduced by Pliny to denote the white of an egg.[123] As far as the brain was concerned, Plato [ca. 428-348 B.C.] likened it to a marrow or pith, and, while Hippocrates did not consider it to contain fat, Aristotle is said to have compared it to a type of fat found in cetaceans.[8] These few examples suggest that the ancients may have possessed more knowledge than has come down to us.

However, they suffered from a lack of understanding of the composition of matter. Their views derived from philosophy rather than experiment, but became so ingrained that they too persisted throughout the Middle Ages. A concept of atoms and a crude kinetic theory of atoms was proposed as the basis of matter [Democritus: ca. 460-370 B.C.], but it failed to displace the more general concepts of four elements: fire, earth, air, and water [Empedocles: 490-430 B.C.], four attributes or qualities: heat, cold, wetness, and dryness [Aristotle], and four humors of the body: blood, phlegm, yellow bile, and black bile [Galen], which were believed to comprise matter and determine its properties through the mediation of a particular essence, or fifth element, hence quintessence [Aristotle].[94]

The ancient concepts of body physiology and nervous system function apparently derived from these beliefs. As outlined by Galen, food stuffs from the alimentary canal were taken as chyle to the liver and refined into blood and "natural spirits" (or "pneuma") which were distributed by the veins to the body. The arterial blood was refined in the heart by air from the lungs via the pulmonary vein to "vital spirits" (or "pneuma") which

ensured heart action and other essential functions. Some "vital spirits" reached the brain through the arteries and were there further refined into "animal spirits" (or "pneuma") which, on distribution through the hollow nerve tubes, produced motion and sensation. The higher functions of the brain in animation of the whole organism were the result of the "psychic pneuma" or "psyche" ("soul"), which originally appears to have been synonymous with "animal spirits," but later, partly through influences of Christianity, became a separate element.[39,42,97] It is these concepts, which came down to the Renaissance virtually intact, that were the points of departure for modern physiology and "animal" chemistry.

FROM THE RENAISSANCE TO THE NINETEENTH CENTURY

This brief outline of the contributions of the scientists of Greece and Rome indicates the type of soil into which the seeds of modern research were sown. The now barren soil was suddenly enriched when monastic domination ceased, communication and trade broadened, and free thought developed. Scepticism and experimentation began to flourish, as the anatomy and physiology of Galen were challenged and Galenic traditions overturned by Vesalius [1514-1564] and Harvey [1578-1657]. Chemistry outgrew alchemy as the better-educated physician entered the field under the stimulus of Paracelsus [1493-1541], Libavius [1540-1616] and van Helmont [1577-1644], who asserted that life was essentially a chemical process and man a complex chemical compound whose health depended upon the correct proportion of its ingredients.[45,94]

From the seventeenth century onward developments came so rapidly that it is no longer possible to consider them simultaneously. Medicine led the way and from it three fields emerged, chemistry, anatomy, and from the latter, physiology (in response to the demand to know how structure functioned). It is here that neurochemistry began to take root. Physiology applied to respiration and digestion became more chemical in its approaches and with the recognition of organic compounds ultimately developed into biochemistry. Physiology applied to the nervous system was soon to become neurophysiology. These two areas of investigation in the seventeenth and eighteenth centuries are the beginnings of our story.

THE RISE OF NEUROPHYSIOLOGY

The stimulus to inquire into the functions of brain, cord, nerves, and muscles was one of the principal results of the work of Vesalius, Harvey, and their contemporaries. During this period most investigators still clung to the Galenic concepts of "animal spirits" and the "psyche" or "soul" as the bases of cerebral and neuromuscular function, but the urge to understand them more precisely soon subjected them to experimental trial. The so-called iatrophysical school developed a highly mechanical explanation of neural function, as exemplified in *Traité de l'Homme*[30] by René Descartes [1596-1650] and *De Motu Animalium*[17] by Giovanni Borelli [1608-1679]. An anatomic basis for cerebral function was proposed by Thomas Willis [1621-1675] in his *De Cerebri Anatome*.[141] Cartesian concepts foreshadowed the demonstration of reflex activity and integrative action, and Willis provided the most complete and accurate account of the nervous system up to that time. But in both instances there was adherence to the idea that the brain was essential to life, motion, and sensation, since it was the seat of the "soul" and the source of "animal spirits" which coursed from it through the nerve tubes to produce muscular contraction by inflation of the muscles.[39,42,45]

The views did not long stand unchallenged. Vesalius and Realdus Colombus had already experimented with decapitation and cerebral ablation. Their example was soon followed by others. As a result of the studies by Boyle, Perrault, Bohn, Vieussens, duVerny, Chirac, and Preston on experimental extirpations and anencephalic monsters, it was recognized that vital functions could persist in the absence of cerebrum and cerebellum, provided the medulla and cord remained intact.[39,42] The proper functions of the brain were revealed because of the interest of the French Academy of Medicine in head injuries. The neurosurgeons concerned, Pourfour du Petit, Lorry, and Saucerotte, established contralateral innervation, cerebral origin of motor and sensory phenomena, medullary localization of the respiratory center, and the role of the cerebellum in coordination and posture.[39,45,97]

The "animal spirits" concept also proved inadequate to account for findings on isolated muscle and nerve-muscle preparations. Francis Glisson [1597-1677] insisted that "irritability" was an inherent property of animal tissues, manifested whenever stimulated directly, by sensory nerves or from the brain, thus distinguishing the central function of perception from sensation. He also showed that muscles did not inflate on contraction, as the "animal spirits" proponents claimed.[39] These studies were extended by Jan Swammerdam [1637-1680] and reached a full elaboration by Albrecht von Haller [1708-1777] and Robert Whytt [1714-1766]. Thus Haller distinguished between sensibility (action of the nerve impulse) and irritability (response or contraction of muscle), or in its later sense, excitability. In his monograph, *On the Vital and Other Involuntary Motions of Animals,*[139] Whytt reported that only a segment of spinal cord was necessary for vital functioning, that movements could be voluntary (willed), involuntary ("spontaneous" or automatic) or mixed, and that muscular contraction occurred under various stimuli – direct, reflex or voluntary.[39]

The Dutch naturalist, Jan Swammerdam,* deserves further mention. In his great *Bybel der Natuur*[121] there is a section entitled: "Experiments on the particular motion of the muscles of the frog; which may be also, in general, applied to all the motions of muscles in Men and Brutes." Some of these experiments, as he depicted them, are reproduced in figure 1. Here are the earliest nerve-muscle preparations, a closed system for visual recording of muscular contraction, and a plethysmographic device to follow changes in muscle bulk with contraction. Such studies confirmed and extended Glisson's observations. Swammerdam could not explain the action of nerve on muscle but posed some very cogent questions:

". . . . it merits particular consideration in what manner the nerve is actually joined to the muscle; how it is constructed in the muscle; what is its course, entrance, middle distribution, and end; as also how it communicates with the moving fibre, and what effect it produces on it; also what that very subtle matter properly is which is undoubtedly conveyed to the muscle through the nerve." [39, p. 46]

A beginning to the answer for these questions came with the studies on "animal electricity." Electric fishes *(Torpedo)* had been known since Roman times and had been studied anatomically by Lorenzini [1678] and John Hunter [1773]. An analogy to these fish appeared to be provided by the Leyden jar, a source of static electricity developed at this time [1745]. The jar was used by Fontana [1757], Caldini, and Galvani to stimulate muscles and nerves and in attempts to stimulate the brain.[45,97] The observation of electrical properties of animal tissues by Luigi Galvani [1737-1798] prompted others to examine this phenomenon. Alessandro Volta [1745-1827] not only recognized the true implications of Galvani's findings but went on to develop the Voltaic pile, the first battery, which provided neurophysiologists with a valuable experimental tool. It is recorded that Valli [1792] attempted to reproduce Volta's pile by connecting the nerves and legs of 14 frogs in series.[94] It is perhaps of more interest that Volta observed:

*Swammerdam was born on February 12, 1637 in Amsterdam. He studied at the University of Leyden under Sylvius and received his Doctor of Physic degree in 1667. Swammerdam's father, an apothecary, was a student of natural history and had a notable collection of insects, fossils, and the like. It is apparent that this influenced Swammerdam in his studies, since he showed much interest in dissection and methods of preparation and preservation of anatomic material, especially insects. His father was entirely unsympathetic with these studies, but Swammerdam persisted. He amassed an outstanding entomologic collection (said to contain 3,000 distinct species of insects, many of them dissected and their life habits traced from hatching), for which the Duke of Tuscany offered him 12,000 florins. In addition, he built up an excellent collection of human anatomic preparations, he was the first to describe the red blood corpuscles, and he carried out studies on cardiac and lung movement, as well as the neuromuscular studies discussed in the text. His *Bybel der Natuur* has been described as "the finest collection of microscopic observations ever produced by one worker" (ref. 42, p. 89). He later published a treatise on bees [1674]. Through his friendship with Stenson (also a pupil of Sylvius), he met the Duke of Tuscany, to whom he demonstrated his frog muscle studies and his insect dissections when the Duke visited him in 1658 (or 1668). He refused the Duke's offer for his entomologic collection since it was made on condition he move to Florence and become a Catholic. His life was complicated by the controversies with his father and a growing preoccupation with religion, influenced by the Flemish mystic and pietist, Antoninis Bourignon. As a result, he retired from the world in 1675 to devote himself to spiritual matters until he died on February 17, 1680 at the age of 43. Swammerdam was "little versed in Latin" so that his publications in Dutch received little attention until Hermann Boerhaave succeeded in purchasing them for 1,500 French florins from Joseph du Verny (who had in turn obtained them from Joubert, the owner, after the death of Swammerdam's heir, Thèvenot). Boerhaave published his work in Latin as the *Biblia Naturæ* in 1738 and an English version, *The Book of Nature; or the History of Insects,* was published in London in 1758, both with biographic data by Boerhaave. With these editions the data he had obtained became general knowledge and influenced all subsequent studies.[39]

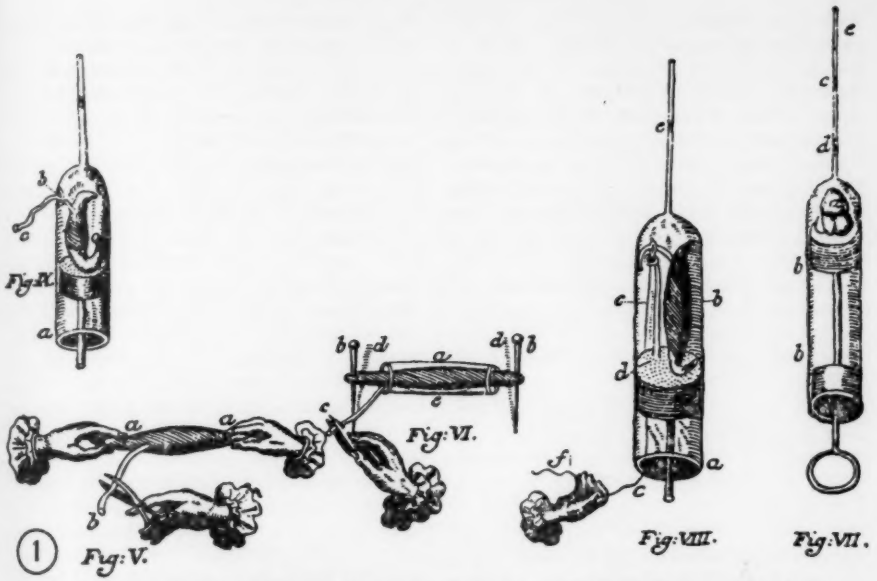


FIG. 1. Swammerdam's figures illustrating his nerve-muscle experiments. Fig. V and VI: measurement of muscle contraction on mechanical stimulation of the nerve; Fig. VII-IX: plethysmographs to demonstrate lack of change in muscle bulk during contraction. [Reproduced from p. 47 of Fearing, F.: *Reflex Action*. Baltimore, Williams and Wilkins, 1930].

"This apparatus . . . . resembles in form more the natural electrical organ of the electric eel . . . . than the Leyden jar and the known electrical batteries . . . . " [38, pp. 117-118]

With this improved source, the electrical excitability of the medulla and spinal cord, as well as nerves and muscles, was demonstrated by Volta, Aldini, and von Humboldt – only the cerebral cortex remained inexcitable due to the relatively weak currents produced by Volta's pile.[97] Galvani maintained that electrical impulses passed from the brain through nerves to all parts of the body, and many of his contemporaries assumed the identity of the nerve agent with electricity.[97]

By the end of the eighteenth century neurophysiology was clearly coming of age. It is important to remember that the investigators of these eras were not specialists but ranged through the whole field of science. They were influenced by clinical observations on the nervous system, by the many advances in chemistry, by the observations of the early microscopists, and by many other developments which cannot be detailed here. Scientific societies and periodicals were begining to appear, facilitating the exchange of ideas and research progress. It is interesting to note that one of the earliest endowed lectureships was provided to the Royal Society by the widow of the seventeenth century physiologist, William Croone:

". . . . one fifth of the clear rent of the King's Head Tavern, in or near Old Fish Street, London, at the corner of Lambeth Hill, 'for the purpose of a lecture and illustrative experiment for the advancement of natural knowledge on local motion, or (conditionally) on such other subjects, as in the opinion of the President for the time being, should be most useful in promoting the objects for which the Royal Society was instituted' . . . . " [108]

The first Croonian Lecture was given in 1738 and they have continued to be a medium for presentation of important developments in nerve and muscle physiology. The bequest returned £ 3 to the society at first, but by 1930 it was yielding £ 56 yearly.[39]

THE BEGINNINGS OF BIOCHEMISTRY

Turning back to take up the other thread of our story, we can consider the factors which led to the emergence of biochemistry, the

studies on respiration and digestion, and the recognition of organic compounds. Much of the chemistry and physiology of the seventeenth and eighteenth centuries was devoted to the problem of respiration and the nature of air and gases in relation to combustion.

Robert Boyle [1627-1691] in his treatise, *New Experiments, Physico-mechanical, Touching the Spring of Air and Its Effects,*[18] described experiments in which he found that a candle flame or a bird could not survive long in a closed vessel under partial vacuum. He commented:

"Paracelsus indeed tells us that as the Stomack concocts Meat, and makes part of it useful to the Body, rejecting the other part, so the Lungs consume part of the Air, and proscribe the rest. So that according to our Hermetick Philosopher . . . . we may suppose that there is in Air a little vital Quintessence . . . . which serves to the refreshment and restauration of our vital Spirits . . . . " [39, p. 55]

Thus air was recognized to be necessary for both life and combustion. In an effort to understand the basis for such findings, studies were undertaken by Hooke, Mayow, Hales, Stahl, Black, Cavendish, Scheele, Priestley, and Lavoisier which led to the recognition of carbon dioxide (the "gas sylvestre" of van Helmont), oxygen, hydrogen, and nitrogen and the correct concepts of combustion and oxidation.

Meanwhile, analogous observations were being made on animals and plants. Robert Hooke [1635-1703], working with Boyle, showed that respiration was not dependent upon lung movement but upon the passage of air through the lungs, demonstrating this by artificial respiration experiments. Hooke stated [1667] that:

". . . . it was not the subsiding or the movelessness of the lungs that was the immediate cause of death, or the stopping of the circulation of the blood through the lungs, but the want of sufficient supply of fresh air." [42, p. 65]

Richard Lower [1631-1691] proved experimentally that air mixed with blood in the lungs to give the latter its red color, while venous blood was dark due to its loss of air. Boyle had already extracted gases from blood under partial vacuum.[42] Stephen Hales [1677-1761] found that a given quantity of air could be breathed if, between expiration and inhalation, the air passed through a rag soaked in caustic potash solution.[94] Oxygen was isolated independently by Scheele [1771, unpublished] and by Priestley [1774]. In his *Experiments and Observations on Different Kinds of Air,*[104] Joseph Priestley [1733-1804] described the reciprocal relationship between plant and animal life – air in which a candle had burned until it went out became respirable and supported combustion again after plants had grown therein for some time.[94] It remained for Antoine Laurent Lavoisier [1743-1794] to put all these observations together. He showed that the "oxygine principle" was taken in by the body and carbon dioxide given out. He found respiration was in every way analogous to combustion, with oxidation in the body resulting in production of heat, carbon dioxide, and water.[42,45]

Even as Lavoisier arrived at the correct concept of respiration, the next advance was being made and published by Lazzaro Spallanzani [1729-1799]* in 1776.[113] In connection with his studies on respiration of warm- and cold-blooded animals and the tolerance of the latter at reduced temperatures to carbon dioxide, Spallanzani studied excised tissues from freshly killed animals. He found that all tissues, including brain, took up oxygen and, in atmospheres of air, hydrogen, or nitrogen, gave off carbon dioxide. He concluded that the actual site of respiration was at the tissue level itself, a view that had to wait a century for its final confirmation and acceptance in the studies of Pflüger [1875].[45,113,115]

Spallanzani also made major contributions to the mechanism of digestion. Fermentation in production of wine and vinegar had been a recognized process since the earliest times. In developing their concepts of a chemical basis for life it was natural that Paracelsus and van Helmont looked upon physiologic processes, including digestion, as due to ferments. Franciscus de la Boe (Sylvius) [1614-1672],

*Spallanzani was born at Scandiano in Lombardy on January 12, 1729. He studied at Reggio and in 1754 became professor of logic, metaphysics, and Greek there. He moved to Modena in 1763 as professor of physics and in 1769 became professor of natural history at the University of Pavia, where he remained until his death on February 12, 1799. Spallanzani was a cleric, holding the title of Abbott, and he travelled widely in the course of his natural history studies. His research covered a wide variety of topics from circulation, respiration, and digestion to spontaneous generation and reproduction.[106,116]

the teacher of Willis, Swammerdam, and Stenson, formulated these ideas by stating [1679] that digestion was a process of fermentation in which saliva and pancreatic juice play principal roles. To this Haller added gastric juice and the aid of bile in fat digestion. Other investigators, notably Borelli, regarded digestion as merely a mechanical trituration process.[42]

In 1752 René de Réamur [1683-1757] reported that his pet kite (a type of hawk) which regurgitated anything it could not stomach, would swallow metal tubes containing food and that when they were returned the food within was softened. He found that gastric juice softened food *in vitro* but, because he did not carry out the experiment at body temperature, complete digestion did not occur. This line of investigation was continued by John Hunter, Stevens, and Spallanzani. In his *De Alimentorum concoctione*[119] Stevens described experiments in which a professional stone swallower was induced to swallow perforated silver spheres containing various types of food. On evacuation he analyzed the contents and proved their conversion into "chyme" or a "kind of pap" without contact with the lining of the stomach or its mechanical action.[123] Spallanzani extended these studies to a variety of species and performed *in vitro* experiments at body temperature which demonstrated the digestive action of gastric juice and, incidentally, its antiseptic properties.[42,114] These observations laid the groundwork for the recognition of enzymes and enzymatic action, which was to come in final form a century later with the studies of Büchner [1896]. Salivin or ptyalin and pepsin were among the first such "ferments" to be isolated and studied early in the nineteenth century.[123]

Parallel with the observations on respiration and digestion, the early recognition of what were later to be called organic compounds occurred. Certain carbon compounds, such as alcohol and sugars, had been known for centuries, but it was Karl Wilhelm Scheele* [1742-1786], who, in his spare time from duties as an apothecary, isolated glycerin, uric acid, and a number of organic acids such as oxalic, tartaric, malic, citric, and lactic acids.[94] It is of interest that his method of isolating the latter as the calcium salts and purifying them with sulfuric acid is still in use for this class of compounds today. Scheele made many other important contributions which marked him as one of the most gifted chemists of all time. The appearance of these new compounds obtained from animal sources, together with the modernization of chemical nomenclature and classifications undertaken by Lavoisier, de Morveau, Berthollet, and Fourcroy, laid the foundations for organic chemistry as developed by Berzelius, Dumas, Chevreul, Liebig, and Wöhler a few decades later.

At the end of the eighteenth century biochemistry as such did not yet exist, but its coming was clearly imminent. The developments in concepts of respiration, digestion, and organic compounds were in large measure responsible for its foundation. Investigators wanted to know how respiration and digestion of foods could result in the support of life and bodily functions and what the processes were that made such support possible. Biochemistry was born from the attempts to answer these questions.

CHEMISTRY OF THE NERVOUS SYSTEM

In comparison with the growth and advances in neurophysiology and general "animal" chemistry, chemical investigations of the nervous system were relatively meager. Most studies in this field were restricted to determi-

*Scheele was born on December 9, 1742 in Stralsund, Sweden. He learned the apothecary's trade in Gothenburg and practiced this trade there between 1737 and 1763. He then worked as assistant to apothecaries in Malmoe (1765), Stockholm (1767), and Uppsala (1770). In 1775 he became owner of the pharmacy in Köping, where he remained until his death on May 21, 1786. Scheele suffered ill health and near poverty and found little appreciation of scientific efforts until Torbern Bergman, professor of chemistry at Uppsala, befriended him and became interested in his work. In addition to the contributions listed in the text, Scheele discovered the elements barium, manganese, chlorine, tungsten, and molybdenum; he distinguished graphite as a form of carbon; and he worked with hydrogen sulfide, arsenic compounds (copper arsenite or Scheele's green and arsine), and Prussian blue (leading to the preparation of hydrogen cyanide). As Moore (ref. 94, pp. 68-71) remarks, Scheele was exceptionally gifted and skillful and must have led a charmed life to be able to survive the discovery of arsine and prussic acid with his primitive laboratory facilities. A few of Scheele's publications may be listed:

On the discovery of uric acid: "Undersøkn, om blåsestenen," *Vetensk. Acad. Handl.* (1776).

On the discovery of lactic acid: "Om mjølk och dess Syra," *Ibid.* (1780).

On the discovery of glycerin: "Røn, betraeffende ett saerskilt sockeraemne uti exprimerade oljor och fetmor," *Ibid.* (1783).

And on the discovery of oxygen (in 1771): "*Chemische Abhandlungen von der Luft und dem Feuer,*" Uppsala and Leipzig (1777).[94,108]

nations of gross chemical composition, reflecting the general interest in anatomy and the nature of body structures. It is understandable that the complexity of the chemical structure of the nervous system proved baffling and resulted in over-long preoccupation with their elucidation. It is not equally clear why the urge to relate nervous function to chemical processes lagged so far behind similar approaches in other areas of animal physiology. Possibly there were more studies than we have on record. Yet these few studies represent the first tentative efforts in the field which has now grown to be neurochemistry.

The seventeenth century pioneers had little to go on but the ancient description of Hippocrates, Plato, and Aristotle. The earliest observations after the Renaissance were apparently carried out by Thomas Bartholin [1616-1680], a Danish anatomist, Nicolas Lémery [1645-1715], a French chemist, and Johann Thomas Hensing [1683-1726], a German physician, plus some observations by Sylvius, Diémerbroeck, Ruysch, Leeuwenhoek, and Burrhus.* [55,111,124] Bartholin described the "temperament" of the brain (cold and wet) and, from its white, soft appearance, greasy feel, and its property of softening and liquefying, he concluded it must consist of fat.[55,124] Leeuwenhoek described the brain substance as formed of small vessels and a type of glassy humor like fat. On distillation, Lémery and others observed that the brain gave forth a large quantity of "limpid phlegm," volatile alkali, and two types of oil, one yellow and buttery, the other black, fetid, and thick like wax. In their anatomic studies Ruysch and others remarked on the "solubility" of much of the brain substance in water.[124]

In 1719 Hensing** published his dissertation, *Cerebri Examen Chemicum ex eodemque Phosphorus singularem omnia inflammabilia accendentem* which appears to be not only the first separate detailed account of chemical analysis of the brain, but also the first report of isolation of a specific substance therefrom. The title page of this work is reproduced in figure 2. This is certainly a very early example of chemical investigation of animal tissues. The emphasis on chemistry as the basis of life and body processes by Paracelsus and van Helmont accounted for the popularity of examining urine, bile, saliva, gastric and pancreatic juices, feces, and later blood with even crude attempts at quantitation.[45] There is little evidence recorded for studies on tissues before this time and even detailed chemical examinations of body fluids came later.† Thus, within the limitations of available records, the work of Hensing and his contemporaries on the nervous system would seem to be indicative of the intense interest in the brain and nerves at that time and to represent an important development in the application of chemistry to body tissues.

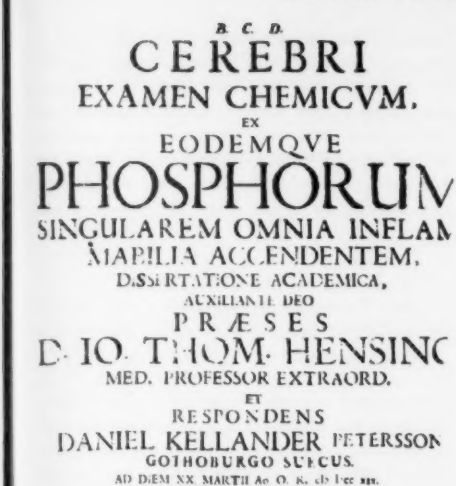
Hensing reported the results of his analyses as follows:

"Divisio itaque feliciter hucusque cerebri peracta est, qua (1) vidimus aquam subtilissimam vulgo spirituosam (quam tamen à consortio subtillisimæ olei immunem non dicere licet (β) oleum à fixiori latice solutum odore admodum volatili, quamvis non adeo grato naribus. (γ) Sal, in quo terra fixis-

*Hensing,[55] Thouret,[124] and Vauquelin[129,131] all cite Burrhus, or von Burrhus, as having analyzed brains and give his principal findings. His identity is in doubt. Poggendorff[103] lists a Guiseppe Francisco Borri (Borro, Burrhus) [1627-1695], alchemist for some years in the service of Frederick III of Denmark, but I have been unable to trace farther than this and am not sure whether these references are all to the same man.

**Only the barest details about this man have been discovered. A biography by J. B. Rüdiger was published in Giessen in 1726, the year Hensing died, but we have been unable to locate a copy. Poggendorff[103] states that Johann Thomas (or Theodor) Hensing was born at Frankfurt am Main on August 30, 1683. He obtained the degree of Doctor of Medicine (probably from the University of Giessen in 1709, since his first publication, *De vitriolo*, appeared in that year from Giessen). He practiced as a "Landphysicus" in Giessen from 1711 to 1717, when he was appointed professor extraordinarius [assistant] of medicine at the University of Giessen. In 1723 he became full (ordinarius) professor "Philosophiæ naturalis chymicæ." He died at Wetzlar on August 27, 1726 at the age of 43. Poggendorff lists eight publications between 1709 and 1725 all on chemical subjects and one more title is listed in the Index of the Surgeon General's Library [Ser. 2, 6:1028 (1901)]. His son, Friedrich Wilhelm Hensing [1719-1745], was an anatomist of some prominence. For Thudichum's summary of Hensing's paper see pages 318-319 of his 1901 edition.[127] Thudichum wrote: "Diese geistreiche Arbeit gibt eine klare Uebersicht des damaligen Zustandes des Wissens über unsern Gegenstand."

†Chemical investigations of muscle were apparently not carried out until 1730 when Claude-Joseph Geoffroy, Jr. reported his studies [*Mem. Acad. des Sci.*, 1730, p. 217 and 1732, p. 17].[126] The sweet taste of diabetic urine was rediscovered by Willis [1679], albumen was found after boiling urine by Dekker [1694], and phosphorus was isolated from urine by Brand and by Kunckel [about 1669].[42,55,103,123] The identification of sugar in diabetic urine and blood came much later [Dobson; 1776], and urea [Rouelle; 1773] and uric acid [Scheele; 1776] were isolated about that same time. Harvey had observed the coagulability of blood serum on heating, and the red cells were described by Swammerdam, Malpighi, and Leeuwenhoek, but the recognition of albumen in serum [Rouelle, 1776], or iron in red cells [Menghini, 1747], and of fibrin and salts in the blood were also much later.[42,123]



B. C. D.

CEREBRI
EXAMEN CHEMICVM,
EX
EODEMQVE
PHOSPHORUM
SINGULAREM OMNIA INFLAM
MABILIA ACCENDENTEM,
DISSERTATIONE ACADEMICA,
AUXILIANTE DEO
PRÆSES
D. IO. THOM. HENSING
MED. PROFESSOR EXTRAORD.
ET
RESPONDENS
DANIEL KELLANDER PETERSSON
GOTHOBURGO SUECUS.
AD DIEM XX. MARTII Ao. O. R. cIↃ IↃCC XIII.
ERUDITORUM EXAMINI SUBMITTENT.

GIESSÆ - HASSORUM,
(2) Typis VID. JO. REINH. VULPII, Acad. Typogr.

FIG. 2. Title page from Hensing's book.

simam aquæ partem compedivit, ut nativam fluiditatem non nisi forte motore accedente, repetere possit. (δ) Terra quoque sicca arida adest, quæ quia tantillum modo (plane enim et omni ex parte exhaustam esse et sibi soli relictam, dubito, quia igne fortissimo vitriariorum, aut solari nondum examinare licuit, ut scirem quid valeat, quid ferre recuset) ex aqueo retinuit, nec aqua solvi nec salinis perfecte subigi, aut igne nostro, liquefieri vel quovis modo mutari potest . . . . annotomus (III) etiam diversitatem texturæ solidarum singularem, mollitiem nempe et facilem divisibilitatem corticis cerebri, et tenacitatem duritiemque medullæ, imprimis corporis callosi, thalamorum duplicis nervi optici et reliquorum nervis eorumque fasciculis et funiculis principium præbentium." [55, pp. 18-19]

His analyses were largely qualitative and no specific mention of albumen was made, but several statements on coagulation of brain indicate that its presence was observed but not recognized as such.

Hensing describes in detail his isolation and identification of phosphorus from the cow's brain. By his tests he was satisfied that it was similar to the phosphorus isolated from urine by Brand and by Kunckel, from feces by Homberg, and from minerals by Balduin and others. This was the "phosphor mirabilis" which Boyle studied in detail and reported in his treatise on noctilucence in 1680. The phenomenon of phosphorescence excited much attention at the time and it is little wonder that many wonderful properties were ascribed to phosphorus. Hensing, in discussing his results, suggested that the substances which he found in brain, including phosphorus, were probably all combined and wondered if phosphorus could be concerned in the functioning of the "animal spirits" contained therein. Thus, in one of the earliest chemical investigations of the nervous system, we see an attempt at synthesis of the findings in terms of function.

The latter objective was often lost sight of in subsequent studies, but the general plan of Hensing's investigation was followed through the time of Thudichum 150 years later. Thouret,[124] Sömmering[111] and Thudichum[125,127] cited a number of similar studies between that of Hensing and Vauquelin's study in 1811. Hamburger reported that from 10,000 parts of gray substance, 8,096 volatilized on drying, whereas only 6,894 volatilized from the marrow.[111, p. 131] Spielmann investigated the ash of the brain and identified iron by its attraction to a magnet.[117] Münch isolated crystalline "Zuckersäure" (saccharic and/or oxalic acid) from brain after treatment with fuming nitric acid.[111, p. 76] Burrhus studied the lipids of the brain and emphasized their similarity to spermaceti.[124,129] Aristotle and Hensing had compared the fat of brains to that found in whales, but Burrhus and later Thouret[124] believed that the solids of the brain were identical with "blanc de baleine" or spermaceti. It remained for Antoine Francois de Fourcroy [1755-1809]* to point out that the substance described by Burrhus and Thouret occurred only in cadaver material and not in fresh brain. Fourcroy showed that "albumen" was a principal constituent of calf, sheep, and human brain, together with mineral salts (ammonium, sodium, and calcium phosphate) in small proportions. He found oily or fatty materials but felt that they represented degradation products from the peculiar "animal matter" of brain. However, he distinguished these lipid materials from ordinary fats and oils and indicated that they must represent a new type or

*Fourcroy was born on January 15, 1755 at Paris. He became a physician and in 1784 was appointed professor of chemistry at the Jardins des Plantes in Paris. He was one of the top chemists of his day and is most noted for his publication with Lavoisier, de Morveau, and Berthollet of *Methode d'une Nomenclature Chimique* [1787]. He died in Paris on December 16, 1809.[103]

species of fatty matter. He also isolated from alcoholic extracts crystals, which he showed were different from those isolated from spermaceti (these were probably cholesterol crystals).[41a,125,127]

During the same period there were a few different observations which we can recognize today as equally, if not more, important, although at the time they occasioned less notice. Stenson had early observed that on compression of the abdominal aorta paralysis ensued distally. Swammerdam and Haller extended these studies. Saunders in his *Treatise on the Liver* wrote,

"The brain receives with relation to its bulk a larger quantity of blood than any other part of the body."[109, p. 58]

and Herdmann quoted,

"Dr. Monro supposes that, not less than a tenth part of the circulating mass is transmitted to the brain."[56, p. 53]

Erasmus Darwin in 1794 recounted the use by millwrights of rotating millstones as a cure for insomnia (cerebral ischemia produced by acceleration).[62] De Rozier reported the effects of entering a brewer's vat during fermentation—sensation of heat, perspiration, violent feeling of suffocation, giddiness, inability to distinguish objects or understand what was said, limb weakness, and purpling of the face.[123, p. 609] Thus, the importance of circulation and oxygenation to function of the nervous system became recognized. Julien Jean César Legallois [1770-1814] in his *Expériences sur le Principe de la Vie* generalizes these concepts:

"Tels sont, en résumé, les principaux faits ques que je fis connaitre en 1809: il en résultait que l'entretien de la vie dans une partie quelconque d'un animal dépendait essentiellement de deux conditions, l'une l'integrité de la portion de moelle epinière correspondante, et de ses communications nerveuses; l'autre la circulation du sang artériel dans cette partie, et par conséquant, qu'il était possible de faire vivre telle partie que l'on voudrait d'un animal aussi longtemps que l'on pourrait faire subsister ces deux conditions . . . . "[77]

The experiments of Astley Cooper in 1836 on the effects of ligating the carotid and vertebral arteries definitely established these concepts for brain, and Brown-Séquard reported similar data for the cord shortly thereafter.[45,62]

Observations on the effects of drugs and toxins upon neural function also began appearing during the seventeenth and eighteenth centuries. Many of the early studies were made by Felice Fontana [1730-1803] in his *Ricerche fisiche sopra il veleno della vipera.*[41] In addition to observing the actions of snake venoms on the central nervous system, Fontana studied the effects of alcohol, laurel, and opium. He noted that, shortly after the application of an opium solution to the frog brain, the animal was unable to move.[45,97] Sömmering[111] wrote that opium in sufficient doses disturbed the activity of the brain, whether taken internally or applied directly to it, in both cases blotting out sensation and consciousness. He listed a number of substances with similar activity, such as strong beer, wine, stramonium, belladonna, and aconite. Sir Humphrey Davy [1778-1829] observed the pharmacologic effects of nitrous oxide, and Sir Benjamin Brodie, Wilson Philip, and others reported studies with "woorara," tobacco infusions, and other toxins.[45,97,123] The practical significance of some of these observations for anesthesia and anticonvulsant action had to await the findings of Long, Morton, and Locock some 50 years later. The functional significances were not recognized until much later.*

THE MODERN ERA

With the contributions of the nineteenth century, we enter into the modern eras for all three fields. It is not feasible to consider the detailed progress in neurophysiology and biochemistry after 1800, and only the highlights pertinent to the growth of neurochemistry will be dealt with. The rapidity of progress in investigations beginning in the nineteenth and extending into the twentieth century is due in large measure to a number of general developments. Many more scientific journals, societies, and meetings were in evidence, true laboratories and new departments in universities were set up, and instrumentation and analytic technics were refined. The cell, which Hook had described in 1665, became an accepted generality in plant and animal economy through the studies of Schleiden and Schwann. Histology and pathology, embryology, and fi-

*In his recent book, *Chemotherapy and the Central Nervous System* (London, Churchill, 1957), McIlwain cites (pages 1-17 *et seq.*) the excellent studies by Snow, Flourens, and others as early as 1847 on the pharmacology and mechanisms of action of ether and analogous compounds. These outstanding investigations tend to be forgotten in later discussions.

nally bacteriology grew rapidly. And through the genius of men like Berzelius and Liebig, organic chemistry and biochemistry became predominant and the new field of physical chemistry appeared. Every area of scientific endeavor received renewed impetus which has persisted to the present time.

NEUROPHYSIOLOGY[19,39,42,45,97]

The great contribution of nineteenth century neurophysiology was the clarification of the functional organization of the nervous system. Sir Charles Bell [1774-1842] was responsible for the final establishment of the sensory function of posterior nerve roots and motor function of anterior roots and for the location of sensory and motor areas of the spinal cord. The latter studies were extended by Legallois, as noted previously. Bell discussed the "nervous circle" which in the hands of Marshall Hall [1790-1858] and Johannes Müller [1801-1858] became the concept of reflex function, anticipated by many eighteenth century workers. These studies later became, in Sir Charles Sherrington's work, the reflex arc and reciprocal innervation. Legallois extended the work of Cruickshank, Bichat, and Brodie to establish the nervous control of respiration and cardiac action. Claude Bernard [1813-1878], Charles Brown-Séquard [1818-1894], Eduard Pflüger [1829-1910], the Weber brothers, and others added observations which culminated in the concepts of the autonomic nervous system elaborated by Walter H. Gaskell [1847-1914] and John N. Langley [1852-1925]. Eduard Weber [1806-1871] and Heinrich Weber [1795-1878] in their study were the first to establish vagus inhibition of the heart. This idea was extended to the central nervous system by Johann Setschenow [1829-1905] and contributed to Sherrington's formulation of the integrative action of the nervous system. Hermann von Helmholtz [1821-1894] measured the velocity of the nerve impulse and showed that muscles were the main source of animal heat. Bernard demonstrated with the use of curare that the muscle was excitable independent of its nerve, and Henry Bowditch [1840-1911] described the "all-or-none" principle and the indefatiguability of nerves.

Contemporary with these studies were those on the central nervous system. Marie Jean-Pierre Flourens [1794-1867] carried out careful observations on the effects of cerebral and cerebellar ablations, establishing the role of the cerebellum in equilibrium and coordination. This approach was extended by Leopold Goltz [1834-1902] in his preparations of decorticate, decerebrate, and spinalless dogs and in similar studies by Sherrington and his associates later.

Perhaps the most significant development was the establishment of electrophysiologic technics. Émile DuBois-Reymond [1818-1896]* was the chief architect of this method. He was aided by the prior observations of Carlo Matteuci [1811-1868] on muscle currents and the development of the galvanometer by Oersted and Nobili. In his book, *Untersuchungen über thierische Electricität*,[33] and later publications, DuBois-Reymond described the demarcation potential, electrotonus, negative variation on tetanization, the action potential of nerve and cord, and the production of acid from muscle after tetanic stimulation. Two other major results came from these studies. Richard Caton [1842-1926] applied DuBois-Reymond's methods to the rabbit brain and observed not only the action potential on sensory stimulation but also spontaneous electrical activity of the electroencephalogram [1875].** He went on to observe the effects of photic stimulation, anesthesia, and death on these potentials. His findings were independently confirmed by Beck and Fleischl, but the full development of the electroencephalogram had to await the invention of the vacuum tube amplifier and the studies of Forbes and Davis and of Berger after 1920. Just a few years before in 1870, Gustav Fritsch [1838-1897] and Eduard Hitzig [1838-1907] finally demonstrated the electrical excitability of the cerebral cortex. David Ferrier [1843-1928] extended their observations, using faradic stim-

*DuBois-Reymond was born on November 7, 1818 in Berlin. He studied at the university of Berlin under Johannes Müller with his fellow student, von Helmholtz. He received his degree in 1843, the subject of his thesis being: *Quæ apud veteres de piscibus electricis extant argumenta.* He succeeded Müller as professor of physiology and director of the physiology laboratories at Berlin in 1858. He died on December 26, 1896 at Berlin.[39,102]

**Brazier[19] points out that Caton was fortunate in choosing the rabbit, since his galvanometer was just sensitive enough to respond to the slow waves of the rabbit's brain. Had he chosen another species he probably would have missed this finding.

ulation, thus laying the basis for modern concepts of cerebral function.

During these many important contributions we should not lose sight of equally important findings being made in the histology of the nervous system. Johannes Purkinje [1787-1869] described the axis cylinder and the presence of nerve cells in the brain [1838]. Valentin observed the continuity of "nerve tubes" from the periphery to the central nervous system and the variability of nerve cell form. Robert Remak [1815-1865] recognized nerve cell processes and non-medullated nerves [1837], and Theodor Schwann [1810-1882] described the neurilemma [1839]. These observations were made possible by the development of the compound microscope by J. J. Lister, but they were usually made on fresh tissue sections without benefit of special technics introduced by later workers. Augustus Waller [1816-1870] reported his studies of peripheral nerve degeneration in 1850; Deiters showed [1865] that each nerve cell possessed an axon and a number of dendrites; Camillo Golgi [1843-1926] introduced metallic stains to demonstrate nerve cells and their processes in brain [1873], and Ehrlich, Ramon y Cajal, Hortega, Nissl, Weigert, and others added more useful stains; and Wilhelm His [1831-1904] demonstrated the embryologic development of neurons from columnar epithelial cells via the neuroblast in 1886. The neuron theory, as summarized by Waldeyer in 1891, became established and in 1897 the concept of the synapse was introduced by Foster.

The picture given by these studies of the fine structure and the function of the nervous system provided Sherrington, Adrian, and the many who succeeded them with an integrated structure, which Sherrington formulated in his *Integrative Action of the Nervous System*[110] and from which neurophysiology today has derived much of its stimulus. This picture would not be complete without recognizing the contributions of the great contemporary neurologists from Broca to Horsley, Jackson and Gowers and their present day pupils.

BIOCHEMISTRY[38,45,94,123]

The chemistry of the nineteenth and early twentieth centuries was dominated by the growth of organic chemistry and biochemistry. It is only recently that physical chemistry and biophysics, which also had their origins in the last century, have reached a par with them. Early in the nineteenth century order was established due to the efforts of Lavoisier, Berthollet, Berzelius, Dalton, and others in the nomenclature, classification, and concepts of combinations and reactions of chemical compounds. The field of organic chemistry resulted primarily from the teachings of the great Swedish chemist, Jöns Jakob Berzelius [1779-1848]. He and his contemporaries were quick to apply general chemical principles to this class of compounds. Methods of organic analysis and synthesis developed rapidly and with them the growth of the coal tar industry. The latter was an important factor for the progress of investigative organic and biological chemistry, since many of the industrial efforts accrued to the benefit of research. Such examples as Chevreul's work on animal fats for the soap industry, Perkin's development of aniline dyes, and attempts to synthesize drugs for treatment of infectious diseases illustrate this point. These developments, together with better apparatus and wider scientific communication, were in large measure responsible for the growth of biochemistry.

Biochemistry originated from two fundamental types of investigations, the elucidation of the nature of organic compounds found in plants and animals and the concept of metabolism, or the assimilation, utilization, functional significance, degradation, and excretion of various compounds by living organisms. We owe much to Justus von Liebig [1803-1873],* professor of chemistry at the Univer-

*Liebig was born on April 5, 1803 at Darmstadt. Liebig's father dealt in artist's supplies and provided him with his first chemical associations. He later trained as an apothecary but was forced to leave when one of his preparations blew up the shop. He then entered the University of Bonn, transferring to Erlangen and then to Paris, where he studied under von Humboldt and Gay-Lussac. He received his doctor's degree from Erlangen and was appointed assistant professor of chemistry at Giessen in 1824 and succeeded to full professorship in 1826. The chemical facilities were very poor, but he secured a deserted barracks in which he oragnized his laboratory (figure 3). This was not the first university chemical laboratory (Lomonosov in Moscow (1748) and Thomson in Glasgow (1817) preceded him in this respect), but it was the first to offer organized laboratory instruction in chemistry. Due to the brilliant teaching and research Liebig's laboratory soon attracted students from all over the world. Liebig was finally forced to curtail his activities and moved to Munich as professor in 1852 to continue in full time reseach. He died there on April 18, 1873. Liebig helped to establish the *Annalen der Pharmacie* in 1832, which subsequently became Liebig's *Annalen der Chemie*, one of the principal media for biochemical communication.[94]

FIG. 3. Liebig's laboratory at Giessen, from a drawing by Trautschold in 1842. [This drawing appears on p. 180 of Moore, F. J.: *A History of Chemistry* (2nd Ed.). New York, McGraw-Hill, 1939; and on p. 117 of Scientific American 196 (Feb. 1957)].

sity of Giessen (1826) and later at Munich (1852), in the initiation of both these concepts. Liebig built on the work of several of his contemporaries. Berzelius did much to introduce the idea of organic compounds and methods for their study through his publications.[14–16] In 1823 Michel-Eugène Chevreul [1786-1889] published his *Recherches Chimiques sur les corps gras d'origine animale*,[23] which was the foundation of modern lipid chemistry. At this same time Jean Baptiste Dumas [1800-1884] was developing important analytic technics for dealing with organic compounds. Liebig and Friedrich Wöhler [1800-1882],* with whom he collaborated closely, devoted much of their efforts to the study of carbon compounds and to the initial syntheses of many of the organic compounds familiar to us. Liebig subsequently became interested in physiologic and agricultural chemistry. He wrote the first treatise on biochemistry[80] and introduced the concept and term of "Stoffwechsel" or metabolism. The laboratory at Giessen where Liebig worked and taught is reproduced from a contemporary sketch in figure 3.

The studies of Liebig and Wöhler in organic chemical structure were continued by many workers. Willy Kühne [1837-1900] introduced the modern concept of enzymes with his work on intermediates of digestion and the effects of pancreatic juice and trypsin isolated therefrom on splitting of proteins. He and Felix Hoppe-Seyler [1825-1895] shared the honors in isolating hemoglobin and Hoppe-Seyler went on to find other respiratory pigments, including chlorophyll, thus anticipating the definitive work of men like Warburg and Keilin some 50 years later. Albrecht Kossel [1853-1927] studied the chemistry of the cell and the cell nucleus, giving us the nucleic acids and some of their purine constituents, as well as recognizing amino acids as the "Bausteine" of proteins. Emil Fischer [1852-1919] extended such work on protein structure and was instrumental in elucidating carbohydrate structures. Indirect contribution to this and other areas of research came from Louis Pasteur [1822-1895] in his studies on stereochemistry, from Michael Faraday, August Kekulé, and Jacobus van't Hoff on valence and bond theories, and from Thomas Graham, Svante Arrhenius, van't Hoff, and Wilhelm Ostwald in

*Wöhler was born in 1800 at Eschersheim. He studied at Marburg and then at Heidelberg under Leopold Gmelin. Gmelin sent Wöhler to continue his studies with Berzelius. In 1824 he became a teacher at the Berlin Technical School and in 1831 was appointed professor at a similar school in Kassel. He was appointed professor of chemistry at the University of Göttingen in 1836 and remained there until his death in 1882. His *Lehrbuch der Chemie* published in 1875 was a popular text for many years. The proximity of Kassel and Göttingen to Giessen must have facilitated his many collaborations with Liebig.[108]

gas diffusion, osmotic forces, and dialysis (distinction of colloids from crystalloids). There were, of course, many other important contributors and contributions.

Turning to Liebig's other main concept, metabolism, it is important to recall the general belief at the beginning of the nineteenth century that only plants could synthesize complex compounds, which must be supplied ready-made to animals, and that there was a distinction between inanimate nature and living matter, which possessed a vital or life force (a vestige of the "spirits" or "pneuma" concepts of Aristotle and Galen). These two beliefs were completely supplanted by the studies of Liebig and Wöhler. In 1828 Wöhler reported the artificial preparation of urea (from heating lead cyanate with aqueous ammonia).[148] Scheele had already synthesized the first organic compound (oxalic acid) much earlier, but Wöhler's artificial urea dealt the death blow to the idea of a vital force and was followed shortly by other similar preparations. At the same time Wöhler published his first paper on the excretion of hippuric acid by an animal fed benzoic acid.[147] In close collaboration with Liebig, Wöhler extended and confirmed these studies in a later paper.[149] Not only did this observation produce the demise of the belief that only plants could synthesize complex compounds, but it resulted in the concept of "Stoffwechsel" or metabolism, which Liebig introduced shortly thereafter. The animal synthesis of uric acid from ammonium carbonate was soon demonstrated, and beginning in 1848 Claude Bernard published a series of papers in which he not only isolated glycogen from the liver but showed its formation there from glucose, and vice versa.[11]

Since these discoveries, metabolism has followed two parallel courses: the detailed steps or course of metabolic reactions and the mechanisms responsible for them. Most of the initial work was done on microorganisms (primarily yeast cells) or on muscle. Studies on digestion by Kühne, Bernard, and others and on fermentation of wines and beer by Pasteur firmly established the enzyme concept. In 1896 Eduard Büchner reported fermentation of sugar by cell-free yeast juice, which he called zymase. This accidental discovery provided the first evidence that enzymes were cellular constituents not functions of whole, living cells. This work was soon extended by Arthur Harden, Carl Neuberg, and others, and the isolation of the first pure, crystalline enzyme (urease) was achieved by James Sumner in 1929. The specificity of enzymes was propounded by Emil Fischer and the concept of coenzymes followed. The identification of the latter as vitamins was materially assisted by the observations of Eijkman, Goldberger, and Funk on the deficiency states of scurvy, pellagra, rickets, and beri-beri, the relief of which Funk believed to be due to "vitamins."

The pioneer observations of Spallanzani on tissue respiration were confirmed and led to the elucidation of the steps in respiratory metabolism during this same period. H. G. Magnus, in a detailed study of blood gases in 1837, found evidence for tissues being the site of oxidation. This was confirmed by direct study of tissues by Pflüger in 1875[102] and by Hoppe-Seyler, using crude apparatus for the observation of gas interchanges by tissues, in connection with his work on respiratory pigments. From Pflüger's work came the concept of the respiratory quotient and the values for various types of foodstuffs, which were extended to intact animals and to man by Voit and Rubner, following pioneer work by Regnault and Reiset.

Considerably before these studies, Scheele had isolated lactic acid from milk and Girtanner had suggested to Richerand in 1800 that muscular contraction was due to the splitting of a chemical substance in muscle.[42] DuBois-Reymond had observed acid liberation from a tetanized muscle [1845], which Kronecker later identified as lactic acid [1871]. Adolph Fick was apparently the first to suggest an association of carbohydrate metabolism with muscle contraction [1867], and in 1882 he demonstrated that chemical energy in muscle can produce work without prior heat production. Claude Bernard pointed out that lactic acid production was an accompaniment of muscle contraction and not, as was then believed, the cause of it [1877]. It remained for Walter Fletcher and Frederick Gowland Hopkins to demonstrate that lactic acid was formed in the absence of oxygen[40] and for Hans Winterstein to show that oxygen was necessary for recovery from anærobiosis.[144]

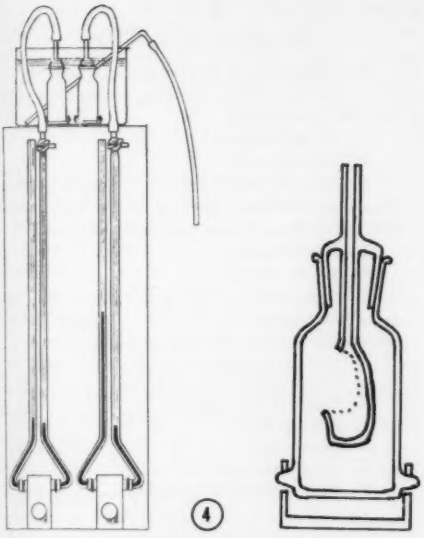


FIG. 4. Drawings of the blood-gas apparatus of Barcroft and Haldane [Reproduced from p. 233 of Barcroft, J. and Haldane, J. S.: A method of estimating oxygen and carbonic acid in small quantities of blood. Journal of Physiology 28:232-240 (1902)].

At the beginning of the twentieth century the metabolism and enzyme studies began to merge rapidly. John Scott Haldane and Joseph Barcroft introduced their apparatus for gas analysis of blood and tissues in 1892. An early model is reproduced in figure 4. Gustav Embden and Otto Meyerhof began their studies on glycolysis, utilizing the enzymatic data from yeast (which Harden, Neuberg, and others were reporting) for application to muscle. Otto Warburg initiated his studies on oxidation and respiratory pigments, drawing on the previous work of Hoppe-Seyler, Vernon, and Batelli and Stern. Warburg developed from the designs of Haldane and Barcroft and of Thunberg and Winterstein the apparatus which is so familiar to us today. With these developments we reach our own period in which the metabolic activities of tissues were rapidly delineated. The details of this recent story have been reviewed by Keilin,[67] Lipmann,[82] Meyerhof,[91] and Warburg[137] and will not be considered further.

Before concluding this discussion, there are a few footnotes to be added. Dumas is credited with discovering the effects of iodine on goiter. Subsequently the field of endocrinology and internal secretions developed, so that by the end of the nineteenth century much basic data had been obtained. Von Mering and Minkowski reported on their studies relating the pancreas to diabetes in 1889, which led to the isolation of insulin by Banting and Best some 30 years later. At about the same time Schäfer and his associates isolated a pressor substance from the adrenals and began work on the pituitary [1894]. In 1866 Gregor Mendel, the abbott of the monastery at Brünn, described his experiments on hybridization of peas. This work did not come to general notice until about 40 years later, but the subject of heredity and natural selection put forth by Darwin, Huxley, Haeckel, and Galton resulted in the emergence of the science of genetics as Mendel's work received recognition. Garrod applied these new findings to biochemistry as early as 1906 in his study of alcaptonuria[46] and evolved the concept of "inborn errors of metabolism" and a biochemical basis for genetics.[47] In 1895 Roentgen described x-rays and the next year Henri Becquerel observed radiations from uranium. The latter discovery was soon expanded in the studies on radium by Pierre and Marie Curie. Thus, the field of radioactivity and isotopes began. The pioneer work of Georg Hevesy in 1923,[58] demonstrating that plants handled a radioactive lead isotope (Thorium B) in an identical manner to stable lead, established the basis for the use of isotopes in biochemical investigations. The basis for another valuable present day investigative tool was laid by the studies of Michael Tswett on the adsorption of substances on calcium carbonate. These were the precursors of our current chromatographic technics. Finally there were the relevant contributions of Sidney Ringer in developing Ringer's solution, of Claude Bernard in his concept of the "milieu interne," of Liebig in considering potassium to be intracellular and sodium extracellular,[86] and of the immunological developments of Jenner, Ehrlich, and Koch.

THE EMERGENCE OF NEUROCHEMISTRY

A. CHEMICAL CONSTITUTION OF THE NERVOUS SYSTEM

During most of the nineteenth century, chemical investigation of the nervous system did not equal the developments in neurophys-

iology and in biochemistry. However, the influences of careful analysis and of organic chemistry became quickly apparent. It is to Fourcroy's pupil, Louis Nicolas Vauquelin [1763-1829] that we owe the application of quantitative analysis to the brain. There were a few prior studies, concerned largely with volatile and non-volatile proportions, but Vauquelin* published the first complete analysis in 1811.[129] His scientific reputation was outstanding and this chemical analysis of the nervous system was widely quoted even after Thudichum. His paper of 1811 was reprinted in French, English, and German journals in 1813.[130,131] The summary from the latter source is reproduced in figure 5. Vauquelin was apparently unaware of previous studies, except for those of Burrhus, Thouret, and Fourcroy, but in the German reprinting J. F. John added footnotes referring to the work of Hensing and a number of other eighteenth century studies. This publication apparently represents the principal source for later reviews of the early literature.

Vauquelin's analysis, summarized in figure 5, approximated those of Thudichum and present day investigators with the exception of the lipid fraction, for which Vauquelin used only alcohol as extractant. Ether was not commonly used for such purposes at the time and chloroform had not yet been synthesized, so that this discrepancy is quite understandable. Vauquelin confirmed previous estimates of water content and established Fourcroy's contention that "albumen" was a major constituent of the solids. He recognized the principal inorganic constituents (potassium, calcium, magnesium, sodium chloride, phosphate, and sulfur) as well as the organic extractives, "osmazome" (a term introduced by Thénard to describe meat extracts or broths). Finally he established the presence of fatty material which was quite distinct from ordinary fats and oils and from the spermaceti and soaps of Burrhus and Thouret.

§. 9.

Allgemeines Resultat.

Die Hirnmasse ist folglich zusammengesetzt:

1. Aus zwei fetten Materien, welches vielleicht eine und dieselbe sind.
2. Aus Eiweifsstoff *).
3. Aus Osmazome.
4. Aus verschiedenen Salzen und unter anderen phosphorsaurem Kalk, phosphorsaurem Kali, phosphorsaurer Bittererde und ein wenig Kochsalz.
5. Aus Schwefel.
6. Aus Phosphor.

Ich halte dafür, so weit es in einer so schwierigen Arbeit möglich ist, dafs diese Substanzen in folgendem Verhältnisse vereiniget sind, nämlich:

1.	Wassers ungefähr	80
2.	Weifser fettiger Materie . . .	4,55
3.	Rother fettiger Materie . . .	0,70
4.	Eiweifsstoffs	7,
5.	Osmazoms	1,12
6.	Phosphors	1,50
7.	Saure, Salze und Schwefels . .	5,15
		100,00.

*) Welcher wahrscheinlich halbgeronnen und im Wasser nicht eigentlich auflöslich ist. John.

5

FIG. 5. Vauquelin's analysis of the chemical composition of human brain. [Reproduced from p. 452 of Vauquelin, L. N.: Analyse des Hirnmarks von dem Menschen und von einigen Thieren. Journal für Chemie und Physik 8:430-460 (1813)]. The original paper of 1811 could not be copied due to the fragile condition of the volume.

Vauquelin also studied the cerebellum, medulla, spinal cord, and nerves. He did not publish his analyses nor did he distinguish cerebral gray from white analytically, but he noted that the medulla and cord contained a great deal more fatty material and less water and "albumen" than the cerebrum, while the cerebellum was similar to the latter. His analysis of nerves was probably the earliest of true analytical studies, since prior reports cited by Sömmering[111, pp. 137-139] were largely qualitative in nature. Vauquelin's results on nerves were less comparable with current data than his central nervous system values, but he recognized their similarity in composition to the latter.

*Vauquelin was born on May 16, 1763 at St. André d'Hébertot. After beginning as a pharmacist in Rouen and Paris, he became "préparateur" (assistant) with Fourcroy in 1783. Vauquelin was appointed apothecary to the Military Hospital at Melun, inspector of mines for the French government, and adjunct in chemistry at the Ecole Polytechnique. In 1801 he was also appointed professor of chemistry at the Collége de France and director of the Ecole spéciale de Pharmacie. Then in 1804 he succeeded Fourcroy as professor of chemistry at the Jardin des Plantes and in 1809 at the Paris Faculty of Medicine. He retired in 1822 and died in the town of his birth on October 14, 1829. Vauquelin was associated with some of the greatest chemists of his era at the center of French chemistry, the Musée d'Histoire naturelle at the Jardin des Plantes in Paris. He worked in all branches of chemistry—minerals, metals, inorganic and organic (both vegetable and animal)—and published extensively.[102] It is of historic interest that Vauquelin and Robiquet isolated and characterized the first amino acid, asparagine (from asparagus), in 1806, four years before Wollaston isolated cystine.[132]

Vauquelin was concerned, as was Hensing nearly a century before, with the natural state of the fractions he had isolated. He was certain that some of the phosphate and the sulfur were combined with the fatty matter and thought some phosphate might also be contained with "albumen." He considered it unlikely that the "albumen" and fatty matter were intimately combined because of their ready separation from each other in water emulsion, where the osmazome and salts remained dissolved. He suggested a simple mixture in which the "albumen" functioned as an "emulsifier" for the fatty material. Vauquelin recognized the potential value of these observations to elucidation of function, but he preferred not to speculate in view of the uncertainties in regard to central nervous system functions.

The impact of this study by Vauquelin is apparent from the numerous investigations of the same nature which followed, culminating in the definitive work of Thudichum in 1884. Despite the controversies and public debates over the results of some of the later work, they all confirmed Vauquelin's basic findings and merely began to fill in the details. Thudichum[125, p. 215] comments, perhaps a bit harshly:

"Vauquelin's data are perfectly correct as far as they go. They furnished an excellent basis upon which the more perfect methods of later times might have built a complete analysis of brain matter. But the later analysts were all deficient in that appreciation of the totality of the subject which alone gives power for exhaustive research. With the one remarkable exception of Couerbe they were men of detail, fishing for some chemical object of passing interest and neglecting their predecessors, as well as the physiological philosophy of the important organ upon which they undertook to operate."

Vauquelin's analyses were rapidly confirmed by J. F. John, using a variety of species (calves, deer, chickens, oxen, crayfish, and man).[65] In 1826 Leopold Gmelin isolated a flaky crystalline fat from brain, which he showed to be identical with "cholestrin" from gallstones.[52] Gmelin and also Kühne isolated a "pulverant brain fat," which Couerbe later characterized as "stéaroconote" (= phosphatides).[125] Jean Louis Lassaigne, who served as Magendie's chemist reported data on retina and optic nerve[76] and on cerebrospinal fluid.[75] He reported the following percentage compositions:

	Water	*Albumen*	*Osmazome*	*Fats*	*Salts*
Horse CSF:	98.180	0.035	1.104	—	0.679+
Retina:	92.90	6.25	—	0.85	—
Optic nerve:	70.36	24.82	0.42	4.40	*

(*Included with osmazome)

CSF salts included: common salt—0.610, carbonate of soda—0.060, phosphate of lime—0.009 and trace of carbonate of lime.

The chemical analysis of brain published in 1834 by J-P Couerbe* was one of the few major contributions between Vauquelin and Thudichum. Couerbe concentrated on the fatty materials of the brain from which he isolated five fractions, summarized as follows:

"Voice le tableau de ces substances:

1° Graisse jaune pulvérulente = stéaroconote, [phosphatides]
2° Graisse jaune élastique = céphalote, [?cephalins]
3° Huile jaune rougeâtre = éléencephol, [a degradation product]
4° Matière grasse blanche de Vauquelin = cérébrote, ["protagon"]
5° Cholestérine."[26, p. 164]

The probable contents of these fractions, according to Thudichum,[125] are indicated in brackets. These studies foreshadowed those of Thudichum, indicating that definite "compounds" might be isolated from the gross brain fractions. Couerbe emphasized this point by publishing elementary analyses for each of his "compounds"— the first example of the use of elementary analysis in brain chemistry. His analysis for cholesterol was remarkably good (C = 84.895; H = 12.099; 0 = 3.006 compared to the theoretical 83.93, 12.00, and 4.07, respectively).

Couerbe's work was remarkable in another way. He felt that such studies ought to help clarify physiologic observations and provide a basis for combatting diseases of the nervous system. In his paper he mentions observations on brains from patients with insanity or idiocy, stating that, whereas the normal percentage of brain phosphorus was 2. to 2.5, the phosphorus from idiots' brains was never over 1.5 and that from lunatics' brains was always 3 to 4.5. He says: "Il faut donc en conclure que cet élément (phosphore) est le principe excitant du système nerveux."[26, p. 191] While the

*No biographic data on Couerbe has been located, although his controversy with Frémy and the Paris Academy of Sciences over his findings of sulfur in his brain fractions must have made him prominent enough at the time.[125,127]

logic of this conclusion may not impress us, it is noteworthy as one of the earliest attempts to relate such experimental data to neuronal function and dysfunction. It is interesting to see a repetition of Hensing's idea of the importance of phosphorus to cerebral functioning. Couerbe's concept is said to have been popularized at the time in the phrase: "Ohne Phosphor, keine Gedanke!"[90]

The ideas proposed by Couerbe awakened little scientific interest. His contemporary, Edmond Frémy [1814-1894], was content to criticize Couerbe's analytical errors and to contribute the isolation of oleophosphoric acid, olein (= palmitin), margarin (= stearin), and fatty acids from cerebral lipids.[43] Nicolas Gobley [1811-1876] extended these studies by obtaining phosphoglyceric, oleic (= palmitic), and margaric (= stearic) acids from compounds he designated as lecithin and cerebrin (= ? cerebrosides), which he showed by analysis to be identical to similar compounds in egg yolk.[53] Numerous studies of this nature followed. W. Müller, in his extensive analyses, isolated creatine, inosite, uric acid (? from xanthine or hypoxanthine), "leucine" (= glycine), and lactic acid.[95] The "protagon" isolated by Liebreich[81] was soon proved to be a mixture of compounds by Diakonow,[31] Strecker,[120] and others, although considerable controversy over it persisted into the twentieth century.[127] Adolph Strecker [1822-1871] demonstrated the composition of lecithin to comprise a fatty acid, phosphoglyceric acid, and choline or neurin.[120] The results of these and many other studies were to advance the understanding of the nature of cerebral lipids, which Fourcroy and Vauquelin had demonstrated to be so different from the usual animal fats.

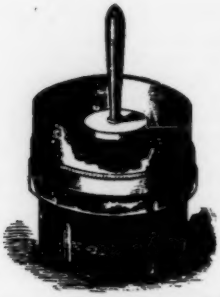
One publication during this period provides a refreshing contrast. In 1854 Ernst Von Bibra* published his *Vergleichende Untersuchungen über das Gehirn des Menschen und der Wirbeltiere*. We have been unable to locate a copy of this work, but judging from Thudichum's brief summary[127] it must have been an excellent study. Thudichum was critical of Von Bibra's method of expressing analytical results and did not rate it very highly. However, it is apparent that Von Bibra was the only one after Vauquelin to examine various parts of the human brain systematically. He noted more water in gray matter than white matter and three to four times as much ether extractable material in the latter compared to the former. His analyses were extended to birds, amphibia, reptiles, and fish, in addition to a variety of mammals. In addition, he examined fetal brains, finding much less ether-extractable material, and observed in the newborn an increase in this fraction together with a decrease in water content. Finally he studied the brains of patients with mental diseases, but Thudichum, while acknowledging these to be of great interest, fails to enlighten us on his findings. The contributions of Von Bibra would seem to be as fundamental as those of Thudichum.

The utilization of analytical and organic chemical knowledge, exemplified by the foregoing studies, reached its culmination in the work of Johann Ludwig Wilhelm Thudichum** carried out between 1865 and 1882. It is interesting that his chemical investigations of the brain were supported by the Privy Council (later the Local Government Board) through its Medical Officer, Sir John Simon, with a view to discovering the effects of typhus on the brain. This objective, as far as we are aware, was never achieved, and in fact Thudichum was insistent that complete knowledge of the normal constitution of the brain was required before one should entertain pathologic aspects. Thudichum made a number of reports of his progress to the Privy Council and Local Government Board and by 1874, in his report for the previous year, was able to present a fairly comprehensive account of his

*Von Bibra was born at Schwebheim on June 9, 1806. He studied for medical and law degrees at Würzburg. Being independently wealthy, he conducted his experiments on natural history (particularly from a chemical standpoint) on his estate and in Nürnberg. He travelled extensively and won several awards for his study on the sickness of match factory employees. He died at Nürnberg, June 5, 1878.[103]

**Thudichum was born in Büdingen on August 27, 1828. He studied at the University of Giessen and at Heidelberg, receiving his medical degree from Giessen in 1851. While at Giessen he was influenced by Liebig to develop a keen interest in chemistry. After serving as a military doctor in Schleswig-Holstein, he emigrated to London in 1853 where he set up a medical practice in otology and rhinology. He was appointed lecturer in chemistry at the Grosvenor Place School of Medicine in 1858 and lecturer in pathological chemistry at St. Thomas' Hospital in 1865. He retired in 1871 to continue work in his private laboratory. During his career he published numerous papers and books ranging from the pathology of urine (1858) and nature of gallstones (1863) to a treatise in 1895 on *The Spirit of Cookery*. He died in London on September 7, 1901.[96, 99, 101]

Apparatus for Filtration by Air-pressure.



Sieve on Fern-Glass and Rubbing Brush for Comminuting Brain.

Fig. 6. Apparatus used by Thudichum for his analyses of brain: On the left a suction-filtration unit; on the right a sieve and rubbing brush for "comminuting brain." [Reproduced from pages 127 and 115 respectively of his 1874 report to the Medical Officer of the Privy Council[125]].

findings.[125] Several pieces of his apparatus were depicted in that report (figure 6). These studies were published in 1884 as *A Treatise on the Chemical Constitution of the Brain* (figure 7) and later in a German translation as *Die chemische Konstitution des Gehirn des Menschen und der Thiere*.[126,127]

Thudichum isolated and characterized most of the major constituents or classes of constituents of the brain which we recognize today. These include the phosphatides (lecithin and cephalins, of which he recognized two, possibly three), sphingomyelin, sulfatides, cerebrosides, cholesterol, and a majority of the trace solutes. He determined the probable composition of the lipid constituents, recognizing phosphatides as combinations of phosphoglyceric acid with fatty acids (oleic, stearic, and palmitic), and a base (choline, ethanolamine, or an unidentified compound), sphingomyelin as a diaminophosphatide derived from sphingosine, and cerebrosides as phosphate-free nitrogenous compounds containing galactose. It is to Thudichum that we owe the flowery Greek terminology which he gave to his newly identified compounds. In addition Thudichum determined the quantitative distribution of the various constituents in gray and white matter of human brain. His summary analysis, reproduced in figure 8, compares favorably with those of today.

With such an analytical basis, it is regret-

A TREATISE

ON

THE CHEMICAL CONSTITUTION

OF

THE BRAIN.

BASED THROUGHOUT UPON ORIGINAL RESEARCHES.

BY

J. L. W. THUDICHUM, M.D.

FELLOW OF THE ROYAL COLLEGE OF PHYSICIANS, LONDON; PRESIDENT OF THE WEST LONDON MEDICO-CHIRURGICAL SOCIETY, ETC., ETC.

LONDON:
BAILLIÈRE, TINDALL, AND COX,
20, KING WILLIAM STREET, STRAND.
1884.

Fig. 7. Title page of Thudichum's treatise, published in 1884.

table that Thudichum did not see fit to apply his knowledge and technics to the problems of function and disease. He writes:

". . . . I have shown the morbid alteration of the nerve marrow in locomotor ataxia, and the occurrence of a kind of glycohæmia to be intimately connected with patholytic changes in substances of the group of cerebrosides. I could go further and unfold, e.g., a chemical connection between the function of the liver and that of the brain . . . . But I have (in early stages of my work) formed the resolution never to propound a generalization on any subject before having proved the validity of all data obtainable by observation or experiment. And I may say that I have not yet found any subject in chemical biology on which, governed by that resolution, I should have liked to proceed to generalization.

The principal reason for this abstension is the circumstance that the data or so-called facts available in chemical biology are as yet too incomplete, and therefore unsuitable for connected treatment except with the aid of hypothesis . . . . "
126, pp. viii-ix

Thudichum concludes his book in a like vein:

"When the normal composition of the brain shall be known to the uttermost item, then pathology can begin its search for abnormal compounds or derangements of quantities. . . . . I believe that the great diseases of the brain and spine, such as general paralysis, acute and chronic mania, melancholy, and others, will be shown to be connected with specific chemical changes in neuroplasm. . . . . In short, it is probable that by the aid of chemistry many derangements of the brain and mind, which are at present obscure, will become accurately definable and amenable to precise treatment, and what is now an object of anxious empiricism will become one for the proud exercise of exact science."126, pp. 259-60

Thudichum's work marked a turning point in the development of neurochemistry. A few investigators continued in this same line of study. Notable among these were Petrowsky,[101] Chevalier,[22] and Halliburton[54] in Europe and the first brain chemists in the United States[24] – Russell Chittenden at Yale, who reported with Kühne on neuro-keratin;[72a] Phoebus Levine at the Rockefeller Institute who refined Thudichum's analytical methods; and Waldemar Koch at Chicago, who, with his sister, Mathilde at the Wistar Institute, initiated detailed studies of the correlation of nervous system development with changes in its chemical composition.[69,70] But until the present era, when chemical architecture of the nervous system is again under study, the emphasis shifted largely to investigation of the chemical bases of metabolism and function.

B. METABOLISM AND ACTIVITY

With the strides made by biochemists in elucidating the chemistry of yeast and muscle metabolism and activity, it is not surprising that their methods and concepts were shortly applied to the nervous system. These applica-

TABLE SHOWING RESULTS OF THE QUANTITATIVE ANALYSIS OF A HUMAN BRAIN.

Weights in grammes; third decimals below 5 omitted, above 5 added as 1 to second decimals. Membranes weighed 55 g.

—	1. Weights with Membranes.	2. Weights without Membranes.	3. Quantities analysed.	4. Albumen.	5. White Matter.	6. W. M. insoluble in Ether.	7. W. M. soluble in Ether.	8. Buttery Matter.	9. Kephalin Total.	10. Myelin Total.	11. Lecithin Total.	12. Cholesterin Total.	13. Inositic.	14. Lactic Acid.	15. Hypoxanthin and Xanthin.	16. Insoluble Extractives.	17. Potassium.	18. Sodium.
Right hemisphere	589	564	465	35·58	18·90	11·63	6·97	21·66	3·24	5·22	5·90	8·93	0·480	0·64	1·28	2·432	0·25	0·49
Left hemisphere	596	570	463	35·06	21·92	12·28	9·65	21·45	—	5·20	—	6·200	0·52	1·00	1·06	2·720	0·29	0·39
Cerebellum	135	129	124	10·94	1·65	1·06	0·22	3·26	1·29	3·76	2·97	1·950	0·650	0·13	0·67	1·490	0·91	0·92
Mesenkephalon and medulla oblongata	84	35	33	2·48	0·64	0·56	0·08	—	0·45	0·23	0·41	1·01	0·06	0·07	—	0·730	0·91	0·98
White tissue	—	—	66	5·70	6·98	4·36	2·42	5·31	0·96	0·14	0·48	2·150	0·14	0·06	—	0·440	0·28	0·04
White tissue for quantitation of water	—	—	9·2	0·80	0·97	0·64	0·33	0·74	0·002	0·02	0·07	0·300	0·02	0·006	—	0·060	0·04	0·056
Grey tissue	—	—	46	3·50	0·30	—	0·20	—	0·15	0·03	0·73	0·900	0·80	0·04	0·10	0·210	0·01	0·09
Grey tissue for quantitation of water	—	—	6·2	0·48	0·04	—	0·04	—	0·02	0·006	0·09	0·120	0·09	0·006	0·01	0·040	0·01	0·01
Loss in operations	—	—	86·6	6·36	4·19	2·26	1·88	4·14	0·63	0·09	1·10	1·340	0·80	0·20	0·21	0·520	0·07	0·07
Totals	1354	1296	1296	101·20	55·48	32·57	21·84	56·76	5·94	15·30	11·75	24·21	1·94	2·14	2·45	9·000	2·78	1·98

8

FIG. 8. Thudichum's table summarizing the results of his analyses. [Reproduced from p. 257 of his 1884 treatise [126].

tions at the beginning of the twentieth century were preceded by some isolated observations.

Between the time of Stenson and of Astley Cooper, the importance of blood supply and oxygen to the functioning of the nervous system had been established. Spallanzani had demonstrated the effect of lowered body temperature on survival under anoxic conditions[115] and Aubert in 1881 reaffirmed these observations.[6] Claude Bernard elucidated the mechanism of carbon monoxide poisoning as a displacement of oxygen by carbon monoxide in the red cell.[13] Gäthgens in 1866 observed that cyanide depressed the oxygen utilization of the body,[48] and in 1888 Geppert showed that this correlated with the inability of cells to remove oxygen from the blood.[51]

Spallanzani had located the site of respiration in the tissues and demonstrated *in vitro* respiration of brain, muscle, and other tissues.[113] Magnus had confirmed the former[42] and Pflüger the latter.[102] Ranke made some early observations on spinal cord in this connection.[106] Barcroft finally established these principles on a modern basis for all tissues, including brain. MacMunn had isolated "myohæmatin" and "histohæmatin" in 1884,[88,89] but this was overlooked until much later.[67] In his experiments with dyes administered *in vivo*, Paul Ehrlich not only demonstrated the blood-brain barrier phenomenon but also observed the decolorization (reduction) of such dyes on tissue activity, which he took to indicate an increased oxygen requirement during such activity.[33] C. A. Herter later repeated these studies with more attention to these effects in neural tissues.[57]

In 1859 Funke observed an acid reaction of the spinal cord during activity and identified it with lactic acid.[44] Ranke[106] and Langendorff[73] found increased lactic acid production on electrical or strychnine stimulation of the cord. These observations were quantitated by Moleschott and Battistini in 1887, using excised samples of resting or stimulated nervous tissue.[93] Their results, in relative values, were as follows:

	Frog CNS	*Rabbit Cord*	*Dog Gray Matter*
Curarized or resting:	100	100	100
Stimulated (electrical or strychnine):	158	173	127

(Determined by titration using phenolphthalein indicator.)

These studies must be among the earliest quantitative metabolic studies on neural tissues.

It may surprise many workers today to find that, when metabolic studies of the nervous system were taken up in earnest, the initial studies were carried out *in vivo* or on whole frog cord-nerve-muscle preparations *in vitro*. Attempts to measure cerebral metabolism by simultaneous carotid and jugular blood sampling were made by Flint in 1862 for cholesterol, and in 1890 by Schtscherbak for phosphate and by de Meyer for sugar.[146, p. 530] Less phosphate was found in jugular blood of normal dogs than in that from dogs under morphine narcosis, while the sugar content of jugular blood was observed to be less than the carotid content.

In 1895 Hill and Nabarro[59] published their studies on the oxygen and carbon dioxide contents of carotid, torcular, and (femoral) venous blood from dogs narcotized with morphine both at rest and with seizures induced by intravenous oil of absinthe. Measurement of blood flow was not attempted and the effect of morphine narcosis was not appreciated. Nevertheless, this study represents the earliest experiment on cerebral respiratory metabolism *in vivo*. Saunders and Mayo in the eighteenth century had both emphasized the relatively large blood flow to the brain, and in 1904 Jensen confirmed this by measuring blood flow in brain and muscle.[64] He reported in the dog a cerebral flow of 138 ml./100 g./min. compared to 12 ml./100 g./min. for muscle. The observations of Hill and Nabarro and of Jensen were combined in the studies by Alexander and his co-workers a few years later, using unanesthetized, narcotized, and photic stimulated dogs.[3–5]. Alexander and Révész[5] found cerebral oxygen consumption of the "resting" dog brain to be 13 cc./100 g./min. or higher (as recalculated by Himwich[60]) compared to a much lower figure for muscle. They found a slightly increased uptake on photic stimulation and a marked decrease during narcosis by various agents. The latter finding accounted for the low values which Hill and Nabarro had obtained. Gayda repeated these studies, utilizing the gas analysis methods of Haldane and Barcroft.[50]. The subsequent developments in this field are well known, starting about 1930 with the observations of Lennox[78] and

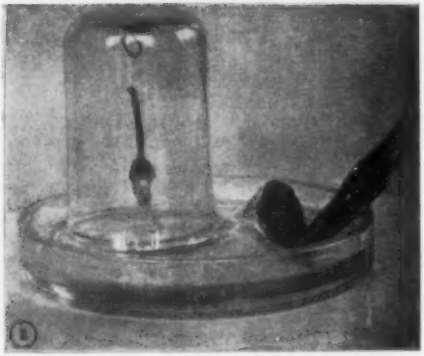


FIG. 9. Photograph of one of Winterstein's isolated frog spinal cord-nerve-muscle preparations. [Reproduced from Fig. 176, p. 444 of Winterstein, H.: Methoden zur Untersuchung des überlebenden Zentralnervensystems. pp. 427-462 in Vol. 5, Part 5B of E. Abderhalden: *Handbuch der biologischen Arbeitsmethoden*. Berlin, Urban and Schwarzenberg, 1938].

Lennox and Gibbs[79] in man and of Himwich and Nahum[61] in the dog. The latter authors found a cerebral R.Q. of 1.0 and oxygen consumption of 14 cc./100 g./min. in their dogs.

Studies *in vitro* kept pace with those *in vivo*. The initial investigations were carried out on isolated frog spinal cord by Baglioni in 1904[7] and Hans Winterstein, beginning in 1907.[142,143] The type of preparation used by Winterstein is illustrated in figure 9. We owe much to Winterstein and his co-workers for the early data they secured on the respiration of the cord, its lactic acid production and glucose utilization, and the effects thereon of temperature, osmotic pressure, ionic composition of media, stimulation, and narcosis. These studies were later summarized by Winterstein in his 1929 review of the subject.[146] The use of breis and slices of nervous tissues was inaugurated by Batelli and Stern in 1908[9] and by MacArthur and Jones in 1917 in the United States.[87] At the same time Warburg was introducing his methods and apparatus for the study of tissue metabolism,[135] so that these technics were quickly adopted by many workers. Beginning in about 1925 the data therefrom, with which we are now familiar, began accumulating. The studies of Loebel in that year are a good example of one of the earliest studies of the metabolism of rat brain slices *in vitro*.[84] Some of his results may be summarized as follows:

	$-Q_{O_2}$	$Q_{CO_2}^{N_2}$	R.Q.
Rat cortex slices with 0.2% glucose	14.7	13.6	0.99
+ 3% ethyl urethane	26%	72%	—
+ 0.02% sodium fluoride	63%	19%	—
No substrate	—	13%	0.86

(Q values = cu. mm./mg. dry wt./hr. % = % of control.)

Coincident with these studies on respiration and glycolysis, the basis for oxygen utilization was being investigated. Ehrlich had found that, on injection of dimethyl-para-phenylenediamine and α-naphthol, an oxidative synthesis of indophenol took place in various tissues.[35] In studying this reaction Röhman and Spitzer[107] concluded that this reaction was due to the catalytic activity of an oxidizing enzyme, which Spitzer in 1897 proposed to be an iron-nucleoprotein compound.[118] Vernon[133] and Batelli and Stern[10] studied this "indophenol oxidase" in a variety of tissues, including brain, and the latter workers studied succinic acid oxidation as well, finding that the oxidase activity catalyzing these reactions was proportional to the respiratory activity of the tissue studied. The studies with indophenol oxidase were one of the precursors of our current histochemical methods and the procedure is still in use in this connection. Warburg[136] believed these reactions to be due to an iron-containing respiratory ferment, analogous to hemoglobin in nature and function, but Wieland[140] and Thunberg[128] had already reported data supporting the idea of hydrogen donors (particularly glucose) and hydrogen acceptors. Ahlgren[1] and Winterstein[145] found evidence for such reactions in nervous tissue. With Keilin's isolation of cytochrome in 1925[66] and Warburg's identification of the role of cozymase in 1932,[138] the links between glycolysis, dehydrogenases, and oxygen requirements began to be understood. Such studies were carried out on a variety of tissue preparations, but the known oxygen and glucose requirements of brain tissue and the early metabolic studies already discussed resulted in simultaneous application of these investigations to brain samples. The preliminary work on the Krebs cycle by Szent-Györgyi, Quastel, and Krebs[72,105,122] and the recognition of the function of phosphocreatine by Meyerhof[91a, 92] brings us to the current era. These subsequent developments have been adequately reviewed elsewhere.[67,71,82,91,137]

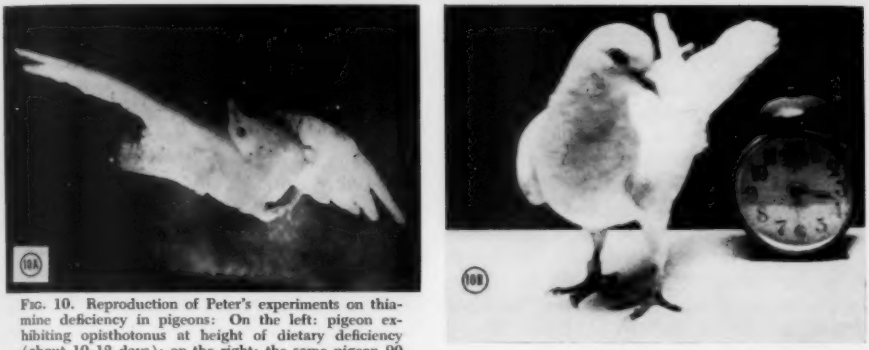


FIG. 10. Reproduction of Peter's experiments on thiamine deficiency in pigeons: On the left: pigeon exhibiting opisthotonus at height of dietary deficiency (about 10-12 days); on the right: the same pigeon 90 minutes after intramuscular thiamine (25 μg.) [Reproduced from unpublished experiments by Tower (1942)].

At this time there was an important application of these studies to the correlation of metabolism in the nervous system with its function. Sir Rudolph Peters and his colleagues, in studies on the effects of polished-rice diets on carbohydrate metabolism of pigeons, were struck by the opisthotonus and other manifestations of neural dysfunction these birds developed.[68] Gavrilescu and Peters[49] observed a marked increase in lactate output by brain samples from such birds, which they termed a "biochemical lesion." Shortly thereafter Meikeljohn, Passmore, and Peters[90a] demonstrated conclusively that the metabolic changes in the brains of deficient birds were due to the B-vitamin deficiency and reported the first evidence for an *in vitro* action of a vitamin in correcting these abnormalities. The administration of the vitamin *in vivo* also rapidly cured the clinical neurologic manifestations, as illustrated in figure 10. The details of the mechanisms involved (the failure of pyruvate oxidation due to deficiency of the thiamine-containing co-enzyme, co-carboxylase) are familiar to us.

These investigations by Peters and his group emphasized two important concepts. The first was the idea of a biochemical lesion which has had numerous applications since, including a number of examples of such lesions involving the nervous system. The second was the fact that study of abnormalities of neural function could help to elucidate metabolic mechanisms. Peters' studies contributed significantly to the understanding of the way in which pyruvate, derived from glycolysis, was utilized for oxidation – the essential link between glycolysis and oxidative metabolism. Here was the practical expression of the philosophy of Hensing, Couerbe, and others that, by chemical investigation of the nervous system, the basis for function and dysfunction could be understood.

Another avenue of research, which bore fruit in this same period, also supported this view. Swammerdam and his contemporaries wondered "what that very subtle matter properly is which is undoubtedly conveyed to the muscle through the nerve."[39, p. 46] Beginning with Fontana, Galvani, and Volta, there had been an increasing identification of electricity with nerve impulses. DuBois-Reymond established the logic of such views, but he himself wondered if the electrical phenomena he demonstrated might not be on a chemical basis.[34] Support for this view came during DuBois-Reymond's lifetime.

Magendie had observed the effects of strychnine on neural activity, and Brodie and Wilson Philip had studied the effects of "woorara," tobacco infusions, and opium. Claude Bernard subsequently demonstrated that curare blocked nerve stimulation of muscle, while the latter still remained directly excitable.[12] The anesthetic properties of ether and nitrous oxide were observed at this time by Long, Wells, and Morton[45] and shortly thereafter Locock accidentally discovered bromide to be an anticonvulsant.[83] By 1882 Albertoni had carried out the earliest experiments on the effects of anticonvulsants, using animals with induced convulsions.[2] The actions of nicotine and pilocarpine on ganglia and muscles were carefully

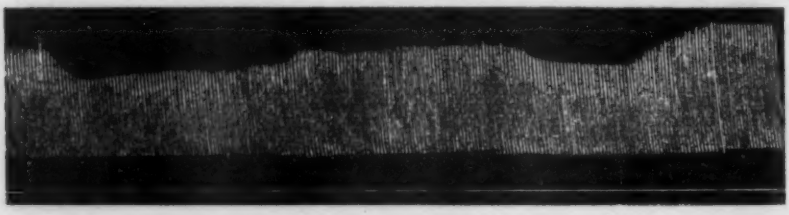
studied in 1890 by Langley,[74] and Schäfer isolated a pressor principle from the adrenal gland the same year.[42,45]

In 1904 Elliott observed that the action of adrenalin closely parallelled that of sympathetic nerves.[37] Dixon made a similar comparison of the action of muscarine with parasympathetic nerve action in 1906,[32] but Hunt and Taveau in the same year remarked on the striking physiologic properties of acetylcholine.[63] Dale extended the latter studies in 1914, observing the "muscarinic" and "nicotinic" actions of acetylcholine and its rapid destruction by tissues.[28] In 1921 Otto Loewi published his first paper on the "Vagusstoff," which very shortly was identified as acetylcholine. One of the records of Loewi's earliest experiments is reproduced in figure 11. Almost simultaneously Walter Cannon reported on the liberation from sympathetic nerves of an adrenalin-like substance, which he termed "sympathin."[21] With the subesquent recognition of acetylcholine and nor-adrenalin as transmitter agents, there was also recognition of the role of electrolytes as the basis for action potentials – a concept anticipated by the membrane studies of Young, Ostwald, Höber, Nernst, and Bernstein[96] and the original suggestion by Liebig of the intracellular position of potassium and the extracellular position of sodium.[86] Thus, the chemical events underlying the neural activities of conduction and transmission began to be appreciated. Like most of the preceding accounts, this story is still incomplete, but the more recent developments are familiar to us all.

NEUROCHEMISTRY AND THE FUTURE

Some aspects in the development of neurochemistry have been covered only briefly, or not at all, and the account has been intentionally ended before the present era. But the origins of and principal factors contributing to neurochemistry are clear. Interest in the nervous system, which began before recorded history, revived and continued to grow after the Renaissance. The applications of chemistry to biologic problems have also grown and the nervous system has shared increasingly in this trend. The origins of neurochemistry were initially concerned with chemical composition or architecture. Hensing and his contemporaries founded this branch of neurochemistry, which was so ably carried on by Vauquelin, Couerbe, von Bibra, and Thudichum. These men did not work from scientific curiosity alone. They felt that such investigations were necessary to the understanding of function and dysfunction.

Biochemistry, the metabolic bases for bodily functions, contributed the second branch of neurochemistry. The origins here began with Spallanzani and his contemporaries, who delineated the primary factors concerned with respiration and digestion, and with Lavoisier, Berzelius, and their colleagues, who established organic chemistry. Liebig and Wöhler com-



1. 2. 3. 2. 4.

Abb. 1. Esculenta. 1. Ringer. 2. Ringer aus 15′ Vagusreizperiode. 3. Ringer aus 15′ Normalperiode. 4. +0,1 mg Atropin.

11

FIG. 11. Loewi's earliest published record of the effect of the "Vagusstoff" on his Straub-heart preparation and its prevention with atropine. [Reproduced from Fig. 1, p. 240 of Loewi, O.: Ueber humorale Uebertragbarkeit der Herznervenwirkung. Arch. f. d. ges. Physiol. 189:239-242 (1921)].

bined these concepts to establish biochemistry and the idea of "Stoffwechsel." The application of biochemistry to the nervous system soon followed with the studies *in vivo* by Hill and Nabarro and Alexander and co-workers and *in vitro* by Winterstein, Meyerhof, and Warburg. Today the foundations established by these investigators have grown into the most active and fruitful branch of neurochemistry, neural metabolism.

The third branch of neurochemistry has derived from neurophysiology. The approaches initiated by Swammerdam and continued by DuBois-Reymond and his contemporaries probably contributed more directly to the development of a chemical basis for neuronal conduction and transmission. Loewi and Cannon inaugurated these studies, which their many successors have established. But the general influence of neurophysiologic progress on the thinking and direction of neurochemical research cannot be overlooked. The common interests of the two fields in neural function make it difficult, if not artificial, to keep their respective contributions distinct.

A number of other important influences have been added to neurochemistry. Pharmacology, biophysics, physical chemistry, pathology, bacteriology have all contributed. Most important of all, clinical neurology, neurosurgery, and psychiatry have profoundly influenced neurochemistry throughout its development. Any incompleteness in acknowledging these and other contributions to neurochemistry merely emphasizes the difficulty in assessing all the factors in the rich heritage which it can claim directly or indirectly.

As we have defined neurochemistry, it now comprises three principal divisions: chemical architecture of the nervous system, neural metabolism, and neural activity. These divisions emphasize the principal areas of interest, but it is obvious that one is not independent of the others. It is also apparent that the complexion of each division is changing with the more recent developments in research methodology and knowledge.

In the field of chemical architecture at least three subdivisions are emerging. The original investigations of composition continue with the application of newer technics of organic and physical methods of isolation, analysis, and characterization. Such work is concerned with delineation of the specific units composing neural structure and their mode of combination into the complexes of neural protoplasm. These investigations merge with those studying how these structures are synthesized and maintained, an aspect closely allied to protein and lipid metabolism. Some investigators in this field are tackling more minute details of architecture – the chemical organization of individual cells and the arrangement of lipid and protein molecules in cell components. Cell fractionation methods, ultramicrochemical procedures, x-ray and ultraviolet absorption technics, electron microscopy, and histochemistry have some of their principal applications in this area. Many investigators using such methods would not consider themselves neurochemists, but they certainly contribute to the growing body of neurochemical data.

The metabolism of neural tissues has many ramifications. Studies *in vivo* provide data on over-all metabolic events in the intact nervous system, correlating with other aspects of bodily metabolism. In addition, there are observations on isolated areas of brain, spinal cord, or nerve *in situ*, which may provide valuable information for bridging the gap between metabolism and function *in vivo* and the aspects of these which can be assessed *in vitro*. Many physiologic technics, notably correlation of electrical phenomena with metabolic events, are being applied here, but there is the potentiality for relating functional responses to cerebral circulation and transfers of compounds from and to the blood across neural membranes and barriers.

Neural metabolism *in vitro* encompasses a wide variety of methods, from the use of cell-containing slices to the use of purified enzyme systems. Such studies also have a variety of approaches. Some are concerned with synthesis and maintenance of structural components and the enzymes contained therein. Others concentrate on cellular energy production which supports all other activities of the cell. Still others involve those aspects of metabolism which initiate, support, and regulate activity of neurons in the reception, conduction, and transmission of impulses. Even though one of these aspects may be emphasized, the metabolic economy of tissues is so

well integrated that all its facets inevitably become part of any such study.

Investigations of the neurochemical bases of central and peripheral neuronal activity (conduction, transmission) are closely related to analogous work in neurophysiology. Traditionally acetylcholine and nor-adrenalin are thought of in this connection, but the potential ramifications are much broader. Other transmitter agents are being proposed, as a result of the stimulus of neurophysiologic studies on central excitation and inhibition. The neurochemists have perhaps neglected the problems of conduction and the functions of electrolytes in activity phenomena, problems with which the neurophysiologists are actively concerned. In terms of cerebral activity the horizons are still broad. There are hormonal aspects, both originating in the nervous system (such as hypothalamic neurosecretion of posterior pituitary hormones) and impinging upon the nervous system from elsewhere in the body. And there are the problems concerned with integrative activity, memory, and mental processes. The "spontaneous" mental disorders and their experimental counterparts induced by various drugs and the role of lipid and protein structure at the synapse or some other portion of the neuron chain in laying down memory traces or stored "information" are isolated examples of neural activity to which neurochemistry needs concerted application.

This brief discussion of the ramifications of neurochemistry today has not been exhaustive, but it indicates most of the principal features of this field. It would require considerable temerity to predict its future. The approaches and problems discussed here are basically similar to those of Elliott[36] propounded nearly a decade ago. Much of the required information and necessary investigations is obvious. It is logical to expect greater advances in the functional aspects of metabolism and structure. These advances will inevitably be reflected in a greater understanding of neural dysfunction. Neurochemistry cannot be expected to solve all these problems. We can only concur with Couerbe when he said:

"Des recherches comparatives faites avec soin éclairciront peut-etre des points de physiologie très importans et donneront des moyens propres à combattre les maladies de cet organe."[26, p. 163]

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Maintenance of the composition of isolated cerebral tissues

Henry McIlwain, Ph.D.

THE PRESENT CONTRIBUTION to this colloquium concerns only one of the many ways in which the nervous system can be studied chemically. It concerns the examination of the isolated cell-containing tissue *in vitro*, in relatively simple aqueous fluids. In appraising the study of the tissue in this condition, it is necessary to query how similar such tissue may be to its state *in vivo*. For this purpose many criteria have been employed and, of those, I will describe some which are biochemical.

One important criterion is comparison of rates, *in vivo* and *in vitro*, of the major energy-yielding reactions of the tissue.[1] Here commonly used incubation fluids, with appropriate stimulating agents such as applied electrical impulses, can yield rates of respiration and glycolysis comparable to those obtaining *in situ*. A second criterion is the metabolic effect of such applied pulses. Metabolic response to pulses shows cerebral tissues, again under commonly used conditions of incubation in oxygenated glucose salines, to be excitable electrically. A third criterion, which is the subject of this communication, is the chemical composition of the isolated tissue. This I will consider in mammalian cerebral tissues prepared as sheets some 0.3 to 0.4 mm. in thickness and incubated in aqueous fluids. The use of nonaqueous fluids in such experiments gives interesting opportunities which will not, however, be discussed here.[2]

Removing the brain and preparing such sheets is obviously a drastic procedure. Many cerebral constituents are susceptible to change in level as a result of manipulations far less severe.[1] Moreover it is such labile constituents, changing rapidly with change in functional state of the brain, which are of especial interest in *in vitro* experiments. Such experiments thus begin with the tissue grossly altered in composition, and the first concern in studying composition *in vitro* must be restoration of *in vivo* levels of labile metabolites.

LABILE PHOSPHATES AND NUCLEOTIDES

Of first importance are the creatine and adenine and related phosphates known to undergo rapid change when the level of functional activity changes in the brain of the living animal. These compounds are degraded in preparing the isolated tissue, but reappear on incubation in simple salines with glucose as the only added organic constituent. Resynthesis, however, is partial only; for example, creatine phosphate, normally at 3 μmoles/gm. brain, falls to 0.3 in preparing the tissue and after 15 minutes at 37° has reached 1.5 μmoles/gm.[3] In seeking an explanation for lack of further resynthesis, creatine itself was found to be lost from the tissue. When 1 m*M* creatine was added to glucose salines, creatine was assimilated to the tissue whose creatine phosphate level rose in two hours to 2.7 μmoles/gm.[4]

Adenine nucleotides presented a similar picture, but their restoration could not be achieved by addition of adenine or of nucleotides; only with adenosine was success achieved.[5] Guanosine restored much of the tissue's lost guanine nucleotides. Two-hour incubation was needed for each to have its maximal effect.

CABOHYDRATE CONSTITUENTS

In preparing the tissue, its glucose and glycogen are largely converted to lactate. On incubating in ordinary glucose salines, lactate and glucose reach normal levels in a few minutes but glycogen only after some hours.[6] The resynthesized glycogen proved unusually stable.[7] Applied electrical pulses which caused conversion of glucose to lactate at 700 μmoles/gm./hr. caused little change in gly-

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cogen. Pulses caused even more rapid changes in creatine phosphate (at some 1500 μmoles/gm./hr. as *in vivo*). For these studies special apparatus was used to sample the tissue[8] at intervals as brief as one second.

OTHER COMPOUNDS

Many contrasts are presented: Cozymase is unexpectedly stable, while ascorbic acid is rapidly lost but again can be restored by incorporation in a glucose saline.[9] Glutathione falls in level and is oxidized on preparing the tissue, but on incubation the balance of oxidized and reduced forms moves towards that obtaining *in vivo*, and glutathione added to glucose salines aids in restoring the *in vivo* levels.[10] Glutamate added to glucose salines increases both the glutamate and potassium ions associated with the tissue toward levels normal *in vivo*.[11,12] Acetylcholine also can be resynthesized by the isolated tissue.[13]

DISCUSSION AND SUMMARY

Thus, although success has not been universal, it has not proved difficult to imitate *in vitro* many aspects of the *in vivo* maintenance of cerebral tissues. This has been done by obvious procedures, such as incubation in the presence of energy-yielding substrates plus precursors of compounds lacking in the tissue. Presumably the process observed to be operating between tissue and artificially-provided salines represents aspects of the normal relationship between tissue and fluids. One then sees the tissue to be remarkably autonomous, taking from its environment essential substances present there at low levels, concentrating them in the tissue, and synthesizing from them further more complex materials. Both concentration and synthesis have been observed to operate only slowly in several instances. This may reflect present experimental limitations, or, equally, may be a genuine characteristic of the processes even as they operate *in vivo*, for in a relatively stable cell population, accretion of material need not be rapid.

When several criteria are available to compare tissue *in vivo* and *in vitro*, the different criteria do not always give similar ratings to the adequacy of given *in vitro* conditions. With isolated tissues, addition of glutamate to a glucose saline increases respiratory rate but lowers creatine phosphate or respiratory response to pulses. The latter two criteria may be judged more relevant than the first, but body fluids contain glutamate. Neither blood plasma nor cerebrospinal fluid may be the most "normal" medium to apply to tissue slices, and probably that most appropriate depends on the aspect of tissue function which it is desired to examine *in vitro*. This is to be regarded as an interim report only on a subject still requiring much study.

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Metabolism of nucleic acids and lipids in brain and in peripheral nerve during experimental demyelination

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THE EXPERIMENTS described in this report are presented to illustrate the type of work which can be done when the technics of chemistry are applied to neurologic problems. The experiments, all of which were carried out *in vitro*, arose as a result of our interest in myelin formation and the problem of demyelination.

BRAIN SLICES

Nucleic acids. Desoxyribonucleic acid (DNA), which is confined to the cell nucleus, occurs in similar concentrations in both white matter and gray matter of brain.[1] On the other hand, ribonucleic acid (RNA), which is largely, but not exclusively, cytoplasmic in origin, occurs in a higher concentration in gray matter than in white matter. This is due to the fact that gray matter contains many more nerve cell bodies than white matter. These cells are much larger than glial cells and contain abundant Nissl material, which is rich in RNA.

When slices of cat brain are allowed to respire in a suitable medium containing glucose and inorganic phosphate labeled with radioactive phosphorus (P^{32}), the RNA of the slices becomes labeled.[2] Much less of the label is incorporated into the DNA. Both the DNA and RNA are also labeled[3] if slices of brain tissue are allowed to respire in a medium containing formate-C^{14}. Typical results of such labeling experiments are presented in table 1.

The *in vitro* labeling of nucleic acids in brain slices is a metabolic phenomenon. It is dependent upon the energy derived from the oxidation of substrates, such as glucose, mannose, pyruvic acid, or lactic acid. However, the labeling is not maintained if the substrate is replaced by certain of the intermediates of the tricarboxylic acid cycle, despite the fact that these intermediates are themselves oxidized. The labeling of nucleic acids in brain slices is also abolished by rendering the conditions anaerobic, by homogenizing the brain tissue, or by the addition of a wide range of metabolic inhibitors.[3–5]

Cholesterol. Cholesterol has long been known to be an important constituent of brain tissue. There is good evidence that it is a component of the myelin sheath.[6] Srere, Chaikoff, Treitman, and Burnstein[7] reported that in brain slices taken from young rats acetate-1-C^{14} is readily incorporated into cholesterol. However, there is no such incorporation when the slices are prepared from brains of adult rats. These findings are in agreement with the results of previous *in vivo* studies which indicate that the cholesterol of the adult brain is very inert metabolically. Table 1 reports the results of a typical experiment in which rat brain slices were incubated in the presence of acetate-1-C^{14}. It can be seen that there was slight labeling of the cholesterol, but that the labeling was insignificant compared with that observed for

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TABLE 1

IN VITRO LABELING OF NUCLEIC ACIDS AND LIPIDS OF BRAIN AND LIVER SLICES INCUBATED WITH RADIOACTIVE PRECURSORS

Precursor	*Fraction Labeled*	*Tissue*	*Specific Activity*
Inorganic P^{32}	RNA	cat brain	22.5[1]
Inorganic P^{32}	DNA	cat brain	2.1[1]
Formate-C^{14}	RNA	rat brain	9.0[1]
Formate-C^{14}	DNA	rat brain	2.9[1]
Acetate-1-C^{14}	cholesterol	rat brain	300[2]
Acetate-1-C^{14}	cholesterol	rat liver (fed)	118,000[2]
Acetate-1-C^{14}	cholesterol	rat liver (fasted)	11,700[2]
Inorganic P^{32}	phosphatide	rat brain	57[1]
Inorganic P^{32}	phosphatide	rat liver	140[1]
Acetate-1-C^{14}	phosphatide	rat brain	880[2]
Acetate-1-C^{14}	phosphatide	rat liver (fed)	3,300[2]
Acetate-1-C^{14}	phosphatide	rat liver (fasted)	260[2]
Glycerol-1-C^{14}	phosphatide	rat brain	2,040[2]
Choline-1,2-C^{14}	phosphatide	rat brain	1,840[2]
Ethanolamine-1,2-C^{14}	phosphatide	rat brain	30,000[2]
Serine-3-C^{14}	phosphatide	rat brain	1,640[2]

[1]Counts/min./μg. P.

[2]Counts/min./mg.

liver slices from either fed or fasted animals (table 1).

Phosphatides. Phosphatides are also important constituents of the myelin sheath.[6] The *in vitro* incorporation of inorganic P^{32} into the phosphatides of brain slices was first reported by Chaikoff and co-workers.[8,9] Strickland[10] confirmed the findings of Chaikoff and associates for slices prepared from cat brain. He showed that the *in vitro* labeling of phosphatide with inorganic P^{32}, like the *in vitro* labeling of nucleic acids, is dependent upon the maintenance of conditions optimal for oxidative phosphorylation. Table 1 shows the results of other experiments in which the incorporation of inorganic P^{32} into the phosphatides of rat brain and liver was studied.

In early experiments it was found that, if the brain was homogenized before incubation, the labeling of phosphatide was reduced to very low levels. However, Kennedy[11] showed that inorganic P^{32} is incorporated into the phosphatides of liver mitochondria. Similar results were found for brain mitochondria[12] and for suitably "reinforced" brain homogenates.[13,14]

The phosphatides of brain slices may be labeled from sources other than inorganic P^{32}. Thus Jedeikin and Weinhouse[15] found that the phosphatide fraction of rat brain slices may be labeled *in vitro* from palmitate-1-C^{14}, and Pritchard[16] showed that the phosphatide may be labeled from acetate-1-C^{14}. Table 1 shows the relative rates of the incorporation of acetate-1-C^{14} into rat brain and liver slices. With liver slices, most of the radioactivity is found in the fatty acid, rather than in the non-fatty acid portion of the molecule.[16,17] However, with brain slices, much less of the radioactivity is confined to the fatty acids.

Table 1 also shows that choline-1,2-C^{14}, ethanolamine-1,2-C^{14}, and serine-3-C^{14} may be incorporated into the phosphatide fraction of rat brain slices. In contrast to the findings reported above for acetate-1-C^{14}, these substances were found to enter the non-fatty acid portion of the phosphatide. When the hydrolysis products of the labeled phosphatides were separated by the method of Dawson,[18] the radioactivity was recovered in the appropriate glycerylphosphoryl base, derived from its parent glycerophosphatide. Further acid hydrolysis of the isolated glycerylphosphoryl bases, with separation of hydrolysis products by paper chromatography, revealed that the bases, but not glycerol, contained the label.

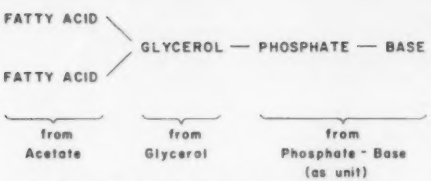

FIG. 1. Scheme of labeling of glycerophosphatides in brain slices.

Glycerol-1-C^{14} also was incorporated into the phosphatides of rat brain *in vitro* (table 1). Most of the radioactivity of the glycerol, like that of the bases, was found in the nonfatty acid portion of the phosphatide. Hydrolysis studies showed that the glycerol, and only the glycerol, of the glycerophosphatide was labeled.

The labeling of glycerophosphatides in brain slices may thus be represented as shown in figure 1. The fatty acid moiety of the glycerophosphatide molecule is labeled from acetate, the glycerol moiety from glycerol, and not from the bases, and the base moiety is labeled from the appropriate base, as such, and not from glycerol. Kennedy[19] has shown that in liver tissue the phosphate base is incorporated into the glycerophosphatide as a unit. Recently Rossiter, McMurray, and Strickland[20] presented evidence that the same is true for brain tissue.

PERIPHERAL NERVE

Fries, Schachner, and Chaikoff[8] demonstrated that inorganic P^{32} could be incorporated into the lipid of dog nerve. Magee and Rossiter[21] confirmed this finding for metabolizing fragments of cat sciatic nerve and they showed that the RNA also was labeled. Further experiments have shown that both the cholesterol and the phosphatide of cat sciatic nerve may be labeled *in vitro* from acetate-1-C^{14} and that the phosphatide may be labeled from glycerol-1-C^{14}, choline-1,2-C^{14}, ethanolamine-1,2-C^{14}, or serine-3-C^{14}.

In general, these results provide evidence that in both brain and peripheral nerve phosphatide may be formed *in situ* from small molecules.

WALLERIAN DEGENERATION

At the present time there is much evidence derived from electron microscopic and other studies (see, for example, paper by Geren[22]) that in peripheral nerve the Schwann cells are responsible for the formation of myelin. If myelin is produced by the Schwann cell, it is of extreme interest and importance to study the metabolism of nerves in which the population of Schwann cells is increased. An increase in the population of Schwann cells may be produced readily in the laboratory by sectioning the nerve. The Schwann cell proliferation, which occurs in that portion of the nerve distal to the point of section, forms part of the classical series of changes known collectively as Wallerian degeneration.

Histologic changes. The histologic changes that accompany Wallerian degeneration are well known and have been described frequently (see Young[23] for a comprehensive review). These include swelling of the axon, followed by later collapse. The nerve soon loses its ability to conduct an impulse. The myelin sheath breaks up into small fragments, with loss of its characteristic optical properties. The Marchi staining reaction becomes positive. The Schwann cells proliferate greatly and the number of macrophages to be seen along the course of the nerve fiber increases. A careful quantitative study of the changes in cell population in a peripheral nerve during Wallerian degeneration was made by Abercrombie and Johnson.[24]

Chemical changes. The chemical changes that accompany Wallerian degeneration are less well known. Some of these were reviewed several years ago by Johnson, McNabb, and Rossiter.[6] With the destruction of the axon, there is a decrease in the activity of certain enzymes, such as alkaline phosphatase,[25] true or specific cholinesterase, and the choline acetylase system. With the destruction of the myelin sheath, there is a disappearance of what we have called the "myelin lipids." Brante,[26] who made a similar study, referred to these lipids as "sheath-typical lipids." It was originally concluded that the myelin lipids comprised free cholesterol, cerebroside, and sphingomyelin, although at the time it was realized that other phosphatides, especially those contained in the classical "cephalin" fraction, might be constituents of the myelin sheath. More recent studies have confirmed that brain diphosphoinositide,[27] phos-

TABLE 2

EFFECT OF NERVE SECTION ON THE RADIOACTIVITY OF THE RNA AND LIPIDS OF FRAGMENTS OF CAT SCIATIC NERVE INCUBATED WITH RADIOACTIVE PRECURSORS

Precursor	*Fraction Labeled*	*Time after Section (days)*	*Mean Concentration*	*Specific Activity*	*Activity (c.p.m./ 100 mg. nerve)*
Inorganic P^{32}	RNA	0	3.9[1]	98[2]	380
		16	18.5[1]	60[2]	1,110
		32	20.8[1]	98[2]	2,000
Acetate-1-C^{14}	cholesterol	0	3.19[3]	130[4]	420
		32	1.15[3]	100[4]	115
		96	0.29[3]	4,960[4]	1,440
Inorganic P^{32}	phosphatide	0	310[1]	8.4[2]	2,600
		16	156[1]	19.0[2]	3,000
		32	78[1]	52[2]	4,100
Acetate-1-C^{14}	phosphatide	0	6.04[3]	780[4]	4,710
		16	2.98[3]	1,360[4]	4,060
		32	1.30[3]	2,390[4]	3,110

[1] μg. P/100 mg. nerve.
[2] counts/min./μg. P.
[3] mg. lipid/100 mg. nerve.
[4] counts/min./mg.

phatidyl ethanolamine, and possibly phosphatidyl serine also may be myelin lipids.[28]

With the appearance of Schwann cells and other cells during the course of the degeneration, there is a considerable increase in the concentration of both RNA and DNA.[27] More RNA appears in the nerve than DNA, so that the RNA : DNA ratio increases, suggesting that the amount of RNA per cell in the degenerating nerve is greater. Together with the increase in the cell population, there is an increase in the activity of certain enzymes, for example, acid phosphatase,[29] pseudo- or nonspecific cholinesterase,[30] and β-glucuronidase.[31]

Labeling of RNA. Magee and Rossiter[21] studied the *in vitro* labeling of RNA from inorganic P^{32} in fragments of degenerating cat sciatic nerve. Table 2 shows that the specific activity of the RNA was not significantly altered as a result of the nerve section, but that the total radioactivity incorporated into the nerve (expressed as counts/min./100 mg. nerve) was greater, because of the large increase in the concentration of RNA.[27]

Labeling of cholesterol. During the first 32 days after nerve section, the labeling of cholesterol in fragments of cat sciatic nerve incubated in the presence of acetate-1-C^{14} was found to be decreased (table 2). However, after 96 days of degeneration, the specific activity of the cholesterol was considerably increased. Because of the decrease in the concentration of cholesterol[32], the increase in the amount of radioactivity that is incorporated into the nerve cholesterol (expressed as counts/min./100 mg. nerve) was not so great as the increase in the specific activity of the cholesterol.

Labeling of phosphatides. With inorganic P^{32} as the source of the radioactivity, the specific activity of the total phosphatide fraction of fragments of cat sciatic nerve was found to be considerably increased in nerves studied 16 and 32 days after section (table 2). However, the decrease in concentration of phosphatide in the degenerating nerve is such that the increase in total radioactivity incorporated into the nerve phosphatide, like the radioactivity incorporated into cholesterol described above, was not nearly so great.

When acetate-1-C^{14} was the source of radioactivity, the results were similar (table 2). There was an increase in the specific activity of the phosphatide fraction of the sectioned nerve, but in this instance there was no increase in the total amount of acetate-1-C^{14} that was incorporated into the nerve phosphatide.

Table 3 shows the results of experiments with other C^{14}-labeled substrates. In all instances there was a considerable increase in the specific activity of the phosphatide fraction in nerves 16 days after section. In the interpretation of these results, it must be remembered that by 16 days the nerve has lost

TABLE 3

EFFECT OF NERVE SECTION ON THE SPECIFIC ACTIVITY OF THE PHOSPHATIDES OF SCIATIC NERVE INCUBATED WITH RADIOACTIVE PRECURSORS

(counts/min./μg. P)

Precursor	*Control*	*Degenerating (16 days)*
[1]Glycerol-1-C^{14}	9	18
[1]Choline-1,2-C^{14}	4	16
[1]Ethanolamine-1,2-C^{14}	52	786
[1]Serine-3-C^{14}	9	196
[2]CMP-P^{32}Ch	2.4	12.7[3]

[1]Fragments of cat sciatic nerve incubated with various precursors.

[2]Homogenates of rat sciatic nerve incubated with CMP-P^{32}Choline.

[3]Studied 13 days after nerve section.

a considerable portion of its myelin lipid[32]. The increases in the specific activity of the phosphatide fraction with glycerol-1-C^{14} and choline-1,2-C^{14} as the source of radioactivity were of the same order as that reported in table 2 for acetate-1-C^{14}. However, with ethanolamine-1,2-C^{14} and serine-3-C^{14} the increases were greater. If these increases are an indication of an increased ability on the part of the nerve to form myelin lipids, as opposed to non-myelin lipids, they would support the suggestion that phosphatidyl ethanolamine and phosphatidyl serine, but not phosphatidyl choline (lecithin), should be included in the list of myelin lipids.

Biosynthesis of glycerophosphatides. Largely as a result of the studies of Kennedy,[19,33] much is known of the metabolic pathways whereby glycerophosphatides are formed. These pathways were worked out for the most part using enzymes derived from chicken liver. Recently, Rossiter, McMurray, and Strickland[20] have provided evidence that lecithin may be formed in brain and nerve by a similar series of reactions. In comparison to chicken liver,[19,33] adult brain and peripheral nerve are rather poor sources of the enzymes concerned with the biosynthesis of the glycerophosphatides. However, using synthetic intermediates labeled with radioactive isotopes, it has been possible to demonstrate that enzymes capable of catalyzing all of the key steps in the synthesis of glycerophosphatides are present in tissue of the nervous system.

The final stage in the biosynthesis of one of the glycerophosphatides, lecithin, is the condensation of cytidine diphosphate choline, a novel intermediate discovered by Kennedy and Weiss,[33] with a D-α,β-diglyceride to form the glycerophosphatide, according to the equation:

$$\text{CDP-choline} + \text{D-}\alpha,\beta\text{-diglyceride} \longrightarrow \text{L-}\alpha\text{-lecithin} + \text{CMP}.$$

Table 3 indicates that, in homogenates of rat nerve, CDP-choline labeled with P^{32} (CMP-P^{32}Ch) was incorporated into the phosphatide fraction. It has been shown with homogenates prepared from rat brain that, in such experiments, the label from the CMP-P^{32}Ch is incorporated into lecithin, but not into phosphatidyl ethanolamine or phosphatidyl serine. In confirmation of the findings of Kennedy and Weiss[33] for liver, the *in vitro* labeling of lecithin in homogenates and mitochondria prepared from rat brain was found to be stimulated by the addition of D-α,β-diglyceride.[20]

In addition, it may be seen from table 3 that, in homogenates of nerves removed from rats 13 days after section, the labeling of lecithin from CMP-P^{32}Ch was greatly increased.

COMMENT

The experiments reported here would appear to provide evidence that phosphatides may be formed *in situ* in both brain and peripheral nerve. For the glycerophosphatides there is evidence that the biosynthesis takes place through a reaction sequence similar to that described by Kennedy[19,33] for chicken liver tissue.

As is well known, there is little *in vitro* labeling of cholesterol from acetate-1-C^{14} in

brain slices prepared from adult rats. The same is true for fragments of cat sciatic nerve. However, the small degree of labeling reported here for both adult rat brain and cat sciatic nerve was a consistent finding. In peripheral nerve the *in vitro* labeling of cholesterol is much greater in nerves removed from animals 96 days after previous section.

When the population of Schwann cells is increased, as evidenced by the increase in the concentrations of DNA and RNA, the labeling of both cholesterol and phosphatide is greatly increased. It seems reasonable to suppose that this increase in the labeling of lipids is an indication of an increase in the potentiality of the nerve for remyelination.

SUMMARY

1. The nucleic acids in slices of rat brain and fragments of cat peripheral nerve, respiring in a suitably buffered medium containing glucose, are labeled from radioactive precursors, such as inorganic P^{32} and formate-C^{14}. The RNA is labeled more rapidly than the DNA.

2. There is a very slight labeling of cholesterol from acetate-1-C^{14} in slices of rat brain and fragments of cat sciatic nerve.

3. Experiments on the labeling of glycerophosphatides from a variety of radioactive precursors provide evidence that in brain and peripheral nerve the phosphatides are formed *in situ* from small molecules. The glycerophosphatides of rat brain and cat sciatic nerve are labeled from inorganic P^{32}, acetate-1-C^{14}, glycerol-1-C^{14}, choline-1,2-C^{14}, ethanolamine-1,2-C^{14}, or serine-3-C^{14}. The fatty acid moiety of the glycerophosphatide is labeled from acetate, the glycerol moiety from glycerol and not from the bases, and the base moiety from the base, as such, and not from glycerol.

4. The pathway for the biosynthesis of lecithin in tissue from the nervous system is similar to that described by Kennedy[19,33] for chicken liver.

5. When the population of Schwann cells in a peripheral nerve is increased by sectioning the nerve, the labeling of the phosphatides and cholesterol is increased.

6. It is suggested that this increase in the labeling of the lipids represents an increase in the potentiality of the nerve for remyelination. This is associated with the increase in the population of Schwann cells.

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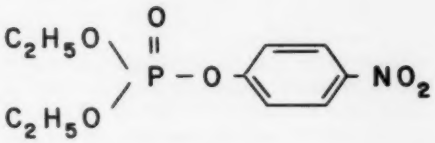
A specific antidote for nerve gas and insecticide (alkylphosphate) intoxication

Irwin B. Wilson, Ph.D.

THIS PAPER will describe some of the theoretical considerations that led to the development of a highly specific and effective antidote for certain alkylphosphates. These alkylphosphates include nerve gases and insecticides, such as diisopropyl fluorophosphate (DFP) and diethyl p-nitrophenylphosphate (Paraoxon) (figure 1). These compounds, although of somewhat diverse structure, have the common feature of containing a labile phosphorus bond and all are potent irreversible inhibitors of many esterases and proteinases. The general structure may be represented by figure 2, where R may be alkoxy or phenoxy, alkyl or acyl, or substituted amino groups, and X may be F, Cl, CN, O-$C_6H_4NO_2$, $OPO(OR)_2$, and so on. The important point is that P-X must be readily hydrolyzable (or the compound converted in living organisms to a new compound which has a readily hydrolyzable bond).



Paraoxon

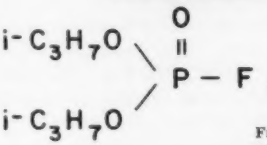


FIG. 1. Structural formulae of DFP and Paraoxon.

(1) D.F.P.

Nachmansohn[1,2] had already demonstrated that the toxicity of these compounds was caused by their ability to inhibit acetylcholinesterase. Can this inhibition be undone?

We had been especially interested in the physical organic chemistry of enzymes and it was particularly interesting in that connection to investigate the inhibitory mechanism of such compounds as these, which were active in concentrations as low as $10^{-10}M$. It turned out that the inhibitory mechanism of these compounds could be very readily explained[3] on the basis of the enzyme theory which had already been developed.[3–5] It will be pertinent therefore to describe briefly the most significant features of this theory. We start with the well established Michaelis-Menten mechanism

$$E + S \rightleftarrows E \cdot S \rightarrow E + \text{products}$$

where E is the enzyme, S the substrate, and E . S is an enzyme-substrate addition compound – the Michaelis-Menten complex. Our problem is to describe the nature of the E . S compound and the nature of the hydrolytic process whereby this compound is transformed to products, with the simultaneous regeneration of the enzyme.

A representation of E . S is shown in figure 3. The active site of the enzyme contains two subsites. One is an anionic site (negatively charged) which attracts the cationic (positively charged) substrate by Coulombic forces and also the methyl groups by Van der Waals forces. The Coulombic force is worth

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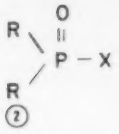

FIG. 2. General structure of alkylphosphates.

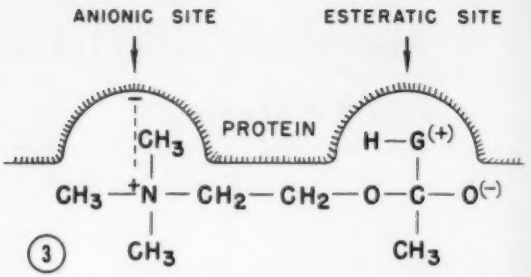

FIG. 3. The acetylcholine-acetylcholinesterase complex, depicting binding at the anionic site and at the esteratic site.

a factor of 30 over an uncharged substrate[3,6] and each methyl group, save one, is worth a factor of 7 in binding.[7] Ethyl acetate, which cannot interact with the esteratic site, would be expected to be bound less readily than acetylcholine by a factor of $7 \times 7 \times 30 = 1500$, as is in fact found to be the case. The other site contains an acidic and a basic group and is represented by the symbol H–G. This site is directly involved in the hydrolytic process. It also contributes to binding by forming a covalent bond between suitable carbonyl groups and the basic group. During the hydrolytic process there is an electronic shift as indicated in figure 4, so that choline is split off and an acetyl-enzyme is formed. In less than a millisecond the acetyl-enzyme reacts with water to form acetic acid and regenerate the enzyme.

The formation of an acetyl enzyme is an important point. Although the hydrolytic process takes place at the esteratic site, the process is *promoted* by interaction with the anionic site.[8]

Now we can return to the mechanism of inhibition by alkylphosphates. These compounds are partially hydrolyzed by the enzyme to yield a dialkyl phosphoryl enzyme* and split off H-X. The basic group which is phosphorylated is the same as is normally acetylated. But unlike the acetyl enzyme, the phosphoryl enzyme does not react with water rapidly; it reacts with water only very slowly. Does the phosphoryl enzyme react with other compounds? Yes, it reacts with a large number of nucleophilic agents,[11] especially hydroxylamine and its derivatives. With 0.1 *M* NH_2OH the enzyme can be reactivated in one-half hour.

A diagram of the phosphoryl enzyme is shown in figure 5. Starting with hydroxylamine, can we design a derivative which will be complementary to the enzyme? Can we construct a molecule which will interact with the anionic site and yet have its nucleophilic function directed at the phosphorus atom? Evidently we need a quaternary structure to interact with the anionic site and to secure

*Phosphorylation was also suggested by Burgen and Hobbiger[9] and demonstrated by Jansen, Nutting, and Balls[10] for chymotrypsin.

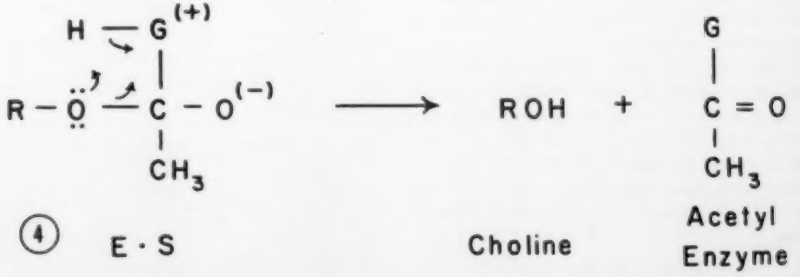

FIG. 4. Electron shifts during hydrolysis resulting in splitting off choline and forming an acetyl enzyme.

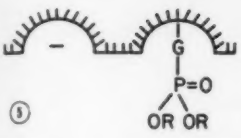

FIG. 5. A dialkyl phosphoryl enzyme resulting from inhibition by an alkylphosphate. The anionic site is vacant and still functional.

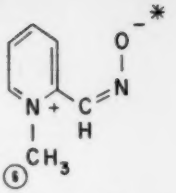

FIG. 6. Structural formula of PAM. The anion (*) is the active agent.

for us the *promoting* effect of such an interaction. The nucleophilic function (the oxygenation of hydroxylamine) must be about 4.5 Å from the quaternary structure. Such a compound is pyridine 2-aldoxime methiodide (PAM)[12] (figure 6). This compound is very active. The enzyme can be regenerated from diethylphosphoryl enzyme in three minutes at 10^{-6} *M*. The importance of the proper orientation of the quaternary nitrogen and the nucleophilic oxygen is shown by the fact that the 3-derivative is inactive and the 4-derivative is considerably less active. But with certain other enzymes the 3-derivative is the most active. (In these cases the absolute activity is millions of times lower).

PAM was then tested *in vivo* with white mice.[13,14] With Paraoxon, DFP, and tetraethylpyrophosphate, a sure lethal dose, followed one or two minutes later by a safe dose of PAM resulted in 100 per cent survival. At high doses where PAM was effective, atropine was without survival value. At still higher doses neither PAM nor atropine was effective, but when administered together they were extremely effective; even with 15 times the sure lethal dose of Paraoxon, 100 per cent of the animals survived.

To round out the theory it was only necessary to show that cholinesterase was reactivated *in vivo*.[15]

Hydroxylamine derivatives, including PAM, can react directly with many of these alkylphosphates. This reaction can also be exploited for protecting animals from these toxic agents and emphasis has been placed upon this method by Summerson and Wagner-Jauregg[16] of the Chemical Warfare Service. In the instances cited above, the direct reaction of PAM with the toxic agent is very slow and this is not the mechanism of protection. For this direct reaction there are a number of compounds which are superior to PAM. These agents, however, are only relatively poor reactivators of the enzyme. In cases of poisoning, where it is believed that the toxic agent is still present in the body, it may well be advantageous to include one of these compounds with PAM and atropine in the treatment.

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Some aspects of the relation of ceruloplasmin to Wilson's disease

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CERULOPLASMIN is a blue, copper-containing plasma globulin which normally constitutes about one-half of one per cent of the total plasma proteins.[1] The present paper will describe briefly the variations in concentration of ceruloplasmin encountered in normal subjects, in patients with Wilson's disease and in unaffected members of their families, and in patients with liver disease, neurologic disease, and schizophrenia. The principal contribution of this paper is the elucidation of the mechanism whereby the hereditary deficiency of ceruloplasmin which characterizes Wilson's disease is brought about.[1]

The plasma concentration of ceruloplasmin can be measured in at least four ways: 1) by quantitative determination of its blue color,[2] 2) by measurement of the oxidase activity which ceruloplasmin imparts to plasma or serum,[3,4] 3) by immunochemical technics,[1,5] and 4) by analysis of plasma or serum copper, since almost all of this is found in ceruloplasmin. The first two methods were used in this work.

METHODS

Spectrophotometric. Blood is drawn from a fasting subject and anticoagulated by adding it to 15 per cent of its volume of 2.5 per cent sodium citrate solution. (This method has not been made to work with serum.) The plasma is separated by centrifugation and clarified by a second and high-speed centrifugation. Using a 5.0 cm. path-length cuvette, the optical density (O.D.) of the plasma is measured in a Beckman DU spectrophotometer at about six wave lengths between 540 and 660 mμ, including 610 mμ. The blue color of the plasma ceruloplasmin is then destroyed by the addition of 0.1 ml. of 10 per cent sodium cyanide solution to each 2.5 ml. of plasma.* About one hour later the optical densities are again read. After correction for the dilution due to the sodium cyanide solution, the difference in optical density (△ O.D.), before and after cyanide, is calculated at each wave length. When plotted against the wave length at which each was read, the △ O.D.s should give a curve similar to that obtained with purified ceruloplasmin.[2,4] The concentration of ceruloplasmin, in mg. per 100 ml., is obtained by dividing the △ O.D. at 610 mμ by 0.0034.[2] Since the relative volumes of blood and anticoagulant are known, the result can be corrected to the value in undiluted plasma.

The accuracy of this method was estimated by adding sufficient purified ceruloplasmin (AK-38S)[4] to ceruloplasmin-free plasma from a patient with Wilson's disease to make the final concentration of ceruloplasmin 66.1 mg. per 100 ml. In two determinations this plasma, with a pH of 7.5, was then found to contain 68.7 and 69.0 mg. of ceruloplasmin per 100 ml., indicating an accuracy of about 4 per cent. The standard deviation of the method in seven sets of duplicate determinations on purified ceruloplasmin preparations was 0.7 mg. per 100 ml.[4]

Enzymatic. Ceruloplasmin is the only component of serum or plasma which catalyzes the oxidation of paraphenylenediamine.[3] Since oxidation of this white substance yields purple-colored compounds, the rate of the reaction is measured in a Beckman DU spectrophotometer, the cuvette chamber of which is kept at 30.0° C. by means of thermospacers.

*We are indebted to Dr. C.-B. Laurell for suggesting the use of sodium cyanide to decolorize ceruloplasmin in plasma more effectively than ascorbic acid does.

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TABLE 1
PLASMA CONCENTRATION OF CERULOPLASMIN IN NORMAL SUBJECTS
(Spectrophotometric)

Initials	*Sex*	*Age*	*Ceruloplasmin (mg. per 100 ml.)*	*Initials*	*Sex*	*Age*	*Ceruloplasmin (mg. per 100 ml.)*
H.S.	M	33	33	A.P.	M	35	25
J.A.M.	F	22	25	H.C.R.	M	23	17
D.C.	M	34	31	D.D.	M	22	32
B.C.H.	M	32	27	J.S.	M	25	18
I.A.	F	40	23	L.B.	M	24	25
M.M.F.	F	22	29	C.M.	M	23	23
W.B.	M	32	16	R.K.	M	22	24
A.S.	F	32	18	M.N.	M	27	16
R.D.S.	F	22	24	M.W.	M	23	26
A.H.	F	27	20	W.W.	M	25	16
F.M.	M	28	29				
						Mean:	23.7

To 1.0 ml. of serum or plasma in a clean, dry 1.0 cm. path-length cuvette are added 2.0 ml. of 0.5 per cent recrystallized paraphenylenediamine dihydrochloride in a solvent of the following composition: 25.0 ml. acetate buffer (pH 5.2, ionic strength 1.2 *M*), and 75.0 ml. of 0.39 per cent acetic acid in 0.9 per cent sodium chloride. The contents of the cuvette are mixed. At accurately timed intervals of about one minute, the optical density of this mixture is read against water at 530 mμ until the increase in O.D. with time becomes constant. This rate of increase is directly proportional to the concentration of ceruloplasmin in the plasma, and, under these conditions (pH of final mixture, 5.00 ± .02; 30.0 ± 0.1° C.), a △ O.D. per minute of 0.0285 corresponds to a serum or plasma concentration of ceruloplasmin of 25 mg. per 100 ml. Autoxidation of the paraphenylenediamine, which can be measured with a serum blank containing no ceruloplasmin, produces a △ O.D. of only 0.001 per minute. The precision of this method, determined from eight sets of duplicates, is such that the standard deviation is 0.0009 △ O.D. per minute.

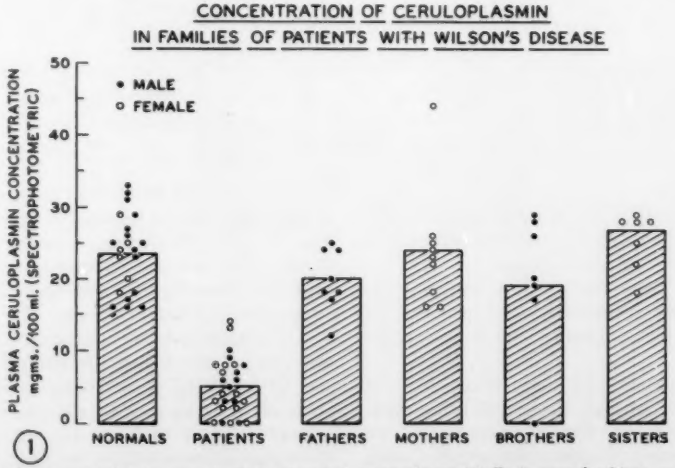

FIG. 1. Plasma ceruloplasmin concentrations determined spectrophotometrically in normal subjects and in patients with Wilson's disease and some members of their families. Height of the bar is the mean.

TABLE 2
PLASMA CONCENTRATION OF CERULOPLASMIN IN PATIENTS WITH WILSON'S DISEASE AND SOME OF THEIR PARENTS AND SIBLINGS
(Spectrophotometric)

Initials	*Sex*	*Age*	*Ceruloplasmin (mg. per 100 ml.)*			
			Patient	*Father*	*Mother*	*Living Siblings*
M.M.	F	15	3	18	22	
E.M.	F	9	2			
D.B.	M	10	10			
R.K.	M	27	3			
B.R.	F	34	8			
Y.R.	F	34	4			
H.K.	F	16	8		25	
L.S.	M	43	3	24		F 28
M.J.S.	F	30	4			M 23 M 20
F.S.	M	39	6			F 28
I.S.	M	43	8			
E.S.	F	25	2			
C.M.	F	15	13			
D.P.	M	20	6			
D.R.	M	27	7			
J.T.	M	16	9		26	M 26 F 17
H.T.	F	40	8			
D.W.	M	11	0	18	16	F 22
R.E.	F	28	7	17	44	F 37
D.S.	F	18	14			
D.F.	M	13	5	24	18	M 19 F 29
S.S.	F	25	3			
D.S.	F	21	0	20	24	M 17 M 29
G.G.	F	13	0	25	23	F 25 F 28 M 0
J.B.	F	17	0	32		
S.I.	F	12	0	12	16	
		Mean:	5	21	24	26

RESULTS

We first studied the concentration of ceruloplasmin, determined spectrophotometrically, in a number of clinical conditions.

Table 1 shows that normal subjects have about 24 mg. of ceruloplasmin per 100 ml. of plasma, with a range of 16 to 33 mg. per 100 ml.

Table 2 and figure 1 show the results in 26 patients with Wilson's disease. Here the mean level is 5 mg. per 100 ml., with several patients possessing no detectable ceruloplasmin. The probability of getting such a difference from the mean of the normal group by chance is much less than 1 per cent, as calculated by the "t" test. Of perhaps even more significance is the lack of overlapping in the values from the normal group with those from these patients with Wilson's disease.

The results in table 2 indicate that neither the parents nor the unaffected siblings over the age of eight years (below which symptoms of Wilson's disease are rarely, if ever, present) appear to have any diminution in their plasma concentration of ceruloplasmin as compared to the normal subjects. Analysis

TABLE 3
PLASMA CONCENTRATION OF CERULOPLASMIN IN PATIENTS WITH LIVER DISEASE
(Spectrophotometric)

Initials	*Sex*	*Age*	*Diagnosis*	*Ceruloplasmin (mg. per 100 ml.)*
W.J.	M	50	Laennec's cirrhosis	27
R.	M	60	Laennec's cirrhosis	39
H.C.	M	44	Laennec's cirrhosis	36
R.F.	M	—	Hepato-splenomegaly	70
C.D.	F	6	Anemia; hepatomegaly	36
M.S.	F	42	Hepato-splenomegaly	29
			Mean:	40

of these data confirms that there is no significant difference between the parent or sibling groups and the normal group. The one sibling with no ceruloplasmin is a younger brother of the very ill girl, G.G. His plasma was first examined when he was ten months old and, like his sister, he was found to have no ceruloplasmin then or at any of several subsequent examinations to his present age of three.

It is of interest to see whether disease of the liver or of the central nervous system other than Wilson's disease is accompanied by decreased plasma ceruloplasmin concentration.

In table 3 and figure 2 the plasma ceruloplasmin levels of six patients with severe liver

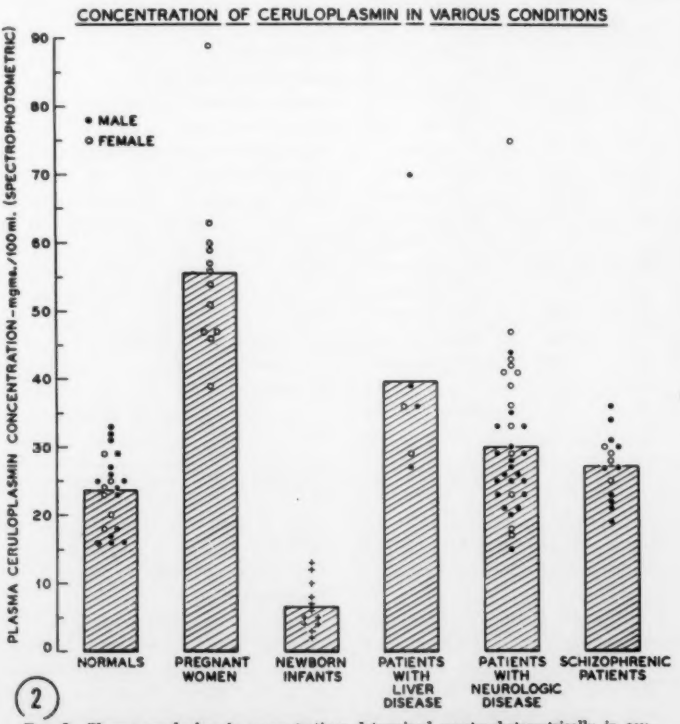

FIG. 2. Plasma ceruloplasmin concentrations determined spectrophotometrically in several clinical conditions. Height of the bar is the mean.

TABLE 4
PLASMA CONCENTRATION OF CERULOPLASMIN IN PATIENTS WITH NEUROLOGIC DISEASE*
(Spectrophotometric)

Initials	*Sex*	*Age*	*Diagnosis*	*Ceruloplasmin (mg. per 100 ml.)*
G.F.	M	6	Phenylpyruvic oligophrenia	25
G.C.	M	50	Jaundice; brainstem disease	23
P.R.	M	13	Involuntary movements	21
H.M.	M	—	Multiple sclerosis	25
D.K.	M	3	Congenital brain abnormality	35
G.LaB.	F	8	Obscure neurologic disease	36
A.P.	M	47	Undiagnosed neurologic disease	29
J.G.	M	44	Undiagnosed neurologic disease	21
A.C.	M	36	Tremors, ? etiology	28
S.H.	F	11	Dystonia	47
H.K.	M	27	Dyskinesia	23
K.S.	F	8	? Cerebral palsy	46
J.G.	M	6	Dystonia musculorum deformans	25
M.M.	F	26	Acute polyneuritis	75
E.B.	M	60	Senile chorea ?	30
A.O.	M	58	Guillain-Barré syndrome	44
A.S.	M	42	Brain tumor	33
G.H.	M	48	Alcoholic polyneuritis	29
D.U.	F	2	Familial leukoencephalopathy ?	43
W.U.	M	5	Cerebral palsy; atonia; mental retardation	33
B.U.	F	4	Cerebral palsy; atonia; mental retardation	39
M.S.	F	4	Polyneuropathy, undetermined origin	29
I.S.	M	34	Encephalitis, ? possible Guillain-Barré syndrome	20
S.M.	M	41	Undiagnosed neurologic disease	26
H.A.	M	62	Undiagnosed neurologic disease	27
H.I.	F	48	Flaccid paraplegia; reticulomyelopathy (possibly Guillain-Barré type)	42
S.F.	F	43	Cerebellar degeneration	23
L.R.	F	—	Huntington's chorea	41
J.T.	F	24	Basal ganglia disease	33
M.E.	F	54	Basal ganglia disease; cerebellar degeneration	18
C.O.	M	55	Undiagnosed neurologic disease	15
D.V.	F	25	Undiagnosed neurologic disease	17
G.F.	M	38	Hereditary ataxia	26
				—
			Mean:	31

*Except for the first two subjects listed in this table, all of these patients were studied in the Neurological Institute of the Columbia-Presbyterian Medical Center through the courtesy of Dr. H. H. Merritt.

disease, by the usual criteria, are shown. These results demonstrate clearly that liver disease does not result in diminished concentration of plasma ceruloplasmin.

In table 4 and figure 2 the plasma ceruloplasmin concentrations of a heterogeneous group of 33 patients with a large variety of diseases of the nervous system are shown. Not one of these patients had a ceruloplasmin level in the range shown by the patients with Wilson's disease. Therefore, neurologic disease does not seem to depress ceruloplasmin concentration.

Figure 2 also shows results in 14 of 20 schizophrenic patients who were hospitalized either at the New York State Psychiatric Institute or at the Bronx Municipal Hospital Center. The mean plasma ceruloplasmin of these 20

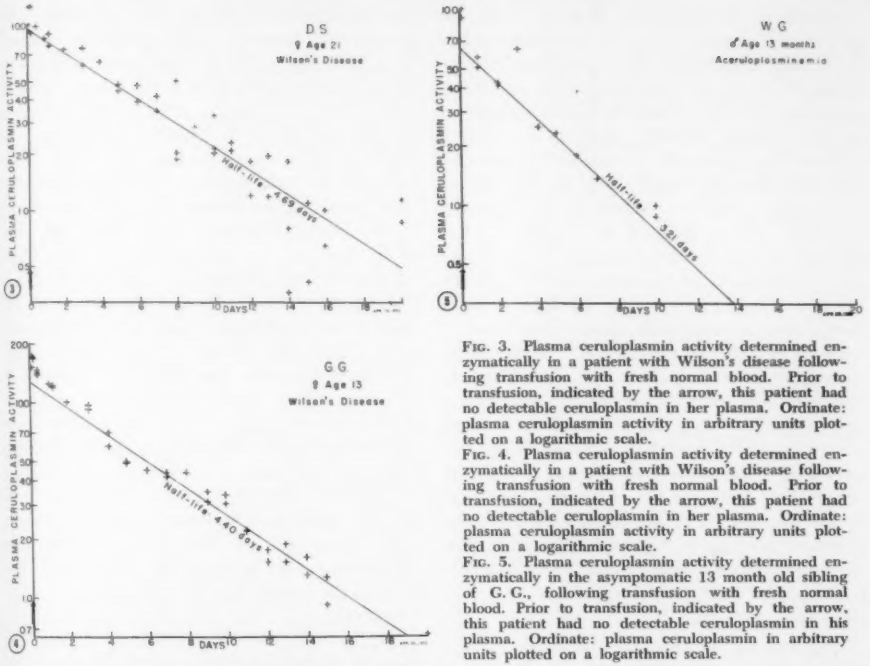

FIG. 3. Plasma ceruloplasmin activity determined enzymatically in a patient with Wilson's disease following transfusion with fresh normal blood. Prior to transfusion, indicated by the arrow, this patient had no detectable ceruloplasmin in her plasma. Ordinate: plasma ceruloplasmin activity in arbitrary units plotted on a logarithmic scale.

FIG. 4. Plasma ceruloplasmin activity determined enzymatically in a patient with Wilson's disease following transfusion with fresh normal blood. Prior to transfusion, indicated by the arrow, this patient had no detectable ceruloplasmin in her plasma. Ordinate: plasma ceruloplasmin activity in arbitrary units plotted on a logarithmic scale.

FIG. 5. Plasma ceruloplasmin activity determined enzymatically in the asymptomatic 13 month old sibling of G. G., following transfusion with fresh normal blood. Prior to transfusion, indicated by the arrow, this patient had no detectable ceruloplasmin in his plasma. Ordinate: plasma ceruloplasmin in arbitrary units plotted on a logarithmic scale.

patients is 28 mg. per 100 ml. This is significantly higher than the mean of the normal group, since the probability of getting such a difference by chance is between 1 and 2 per cent, as calculated by the "t" test. The overlapping of results from the normal group (range: 16 to 33 mg. per 100 ml.) with those from the schizophrenic group (range: 19 to 37 mg. per 100 ml.) is so great, however, as to make the plasma concentration of ceruloplasmin essentially useless in determining whether an *individual* is normal or schizophrenic.[13] The clinical value of the difference between normal and schizophrenic groups is even further lessened by the fact that the difference from normals found in both the patients with liver disease and neurologic disease has an even greater level of significance than $P = 0.01$, as calculated by the "t" test. These findings are of interest in view of a recent report which suggested that "ceruloplasmin activity" might be elevated in schizophrenic, manic depressive, and senile psychoses.[6]

Figure 2 also shows that, although pregnant women have very high plasma concentrations of ceruloplasmin at term, their newborn infants have levels almost identical to the average in patients with Wilson's disease.[7,8] The only other significant condition in which hypoceruloplasminemia occurs is the nephrotic syndrome where ceruloplasmin is excreted in the urine.[5]

The foregoing results constitute evidence that a specific (and presumably inherited[14]) deficiency of ceruloplasmin is seen in patients with Wilson's disease. We were interested in determining whether this deficiency resulted from a diminishd rate of synthesis or an excessive rate of destruction of the protein in comparison to the normal subject. For this purpose, incomplete exchange transfusions were performed in three subjects who had no detectable plasma ceruloplasmin. Two of these individuals were suffering from severe Wilson's disease; the third was the younger brother of one of these, who has already been referred to, and who was asymptomatic.

Enough normal blood was transfused into these subjects to raise their plasma concentrations of ceruloplasmin sufficiently so that meas-

urement of ceruloplasmin activity could be performed enzymatically, as described above. Following transfusion the enzymatic ceruloplasmin activity of the serum was measured daily and the rate of fall of the concentration of the protein thereby determined. When plotted logarithmically a reasonably straight line was obtained from which the half-life of the protein could be calculated. In figure 3, this line, drawn through the points by the method of least squares, corresponds to a half-life of 4.69 days. In the second patient, where the precision of the results seems somewhat better, the ceruloplasmin appeared to have a half-life of 4.40 days (figure 4). In the third aceruloplasminemic subject, the asymptomatic boy who was then 13 months old, ceruloplasmin appears to have had a half-life of 3.21 days (figure 5), but the precision of this experiment is the poorest of the three. Because of the time required to approach equilibrium in the distribution of the administered ceruloplasmin, serum ceruloplasmin concentrations of the first two days were omitted in calculating the half-life of the protein.

DISCUSSION

The finding of a specific deficiency of ceruloplasmin in a patient who is not newly born and who is not suffering from the nephrotic syndrome is probably a necessary and sufficient condition for the diagnosis of Wilson's disease. Although this illness appears to be inherited in an autosomally recessive manner,[14] neither the parents, who are, of course, carriers, nor the unaffected siblings of patients can be detected by means of plasma ceruloplasmin determinations since both groups possess normal concentrations of this protein.

It appears that normal ceruloplasmin has a half-life of about four days in these three subjects who possess essentially no ceruloplasmin. Can this fact be of use in answering the question whether the deficiency of ceruloplasmin in patients with Wilson's disease is due to excessive destruction or diminished synthesis of the protein? Let us assume, first, that the virtual absence of this protein is due to a much greater rate of destruction of the protein than normal. The rate of synthesis of the protein is assumed to be at least as great as the normal. Since these patients have less than 1 per cent of the normal concentration of ceruloplasmin, the half-life of ceruloplasmin in normal subjects would have to be at least 400 days, since it must be a hundred times greater than in these patients in the face of the same synthetic rate in the two groups. Such a half-life for a plasma protein is so large in comparison to those of plasma proteins that have been studied as to be very unlikely.[9,10] The alternative explanation for the absence of ceruloplasmin in these patients remains, that is, that their rate of synthesis of this protein is much less than normal. In the absence of evidence that patients with Wilson's disease synthesize an abnormal ceruloplasmin which is broken down with a half-life of about an hour, it seems reasonable to conclude that deficient synthesis of ceruloplasmin is the cause of the low concentrations of ceruloplasmin in this condition.

The concept of Wilson's disease as a specific, inherited deficiency of a plasma protein makes it closely analogous to such disorders as congenital agammaglobulinemia and congenital afibrinogenemia.[11,12] The defect in these diseases, too, seems to lie in the synthesis, rather than in the destruction of the protein.

SUMMARY

We have presented the results of determinations of the concentration of the plasma protein, ceruloplasmin, in various clinical conditions. These show that Wilson's disease is characterized by a marked deficiency or absence of the protein, but that neither liver disease nor neurologic disease of various other kinds is associated with diminished concentrations of ceruloplasmin. In fact, in both of these latter groups the mean value for plasma ceruloplasmin was significantly ($P < 0.01$) greater than the mean of the normal group. Although the mean concentration of plasma ceruloplasmin in a group of schizophrenic patients was slightly higher than the mean of the normal group, determination of the concentration of ceruloplasmin seems to be of no value in differentiating schizophrenic from normal individuals. Studies performed in patients with Wilson's disease indicate that their deficiency of ceruloplasmin is a consequence of impaired synthesis of the protein.

Professor John W. Fertig and Dr. Agnes Berger of the Columbia University School of Public Health and Administrative Medicine kindly aided us in the statistical analysis of all the results of this paper. Dr. Raymond M. Simon assisted us in the study of patients in the Bronx Municipal Hospital Center.

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Muscle proteins in dystrophy

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PROTEINS prepared from dystrophic muscles have been studied in the ultracentrifuge in an attempt to characterize these proteins by comparison with similar preparations from normal muscle. Preliminary to these studies an extraction procedure giving high yield was required because of the small size of the samples obtained on biopsy. A slight modification of the procedure of Hajdu and Szent-Gyorgyi[1] gave better yields than extraction with potassium chloride or with Weber-Edsall fluid.

Muscle obtained on biopsy was stored in the deep freeze. The procedure was carried out with 2 gm. samples homogenized with 2 ml. of a solution containing 1.2 *M* KI, 0.25 *M* $NaHCO_3$, 0.06 *M* $Na_2S_2O_3$, 2 m*M* adenosine triphosphate (ATP), and a trace of ascorbic acid at approximately pH 9. The homogenate was diluted with a solution containing all the above constituents at one-half of the concentrations mentioned. The muscle residue was thrown down using the high speed attachment of the centrifuge, and the myosin-containing supernatant diluted to 0.05 *M* KI at pH 7 (at room temperature). The precipitate was washed with 0.05 *M* KCl and dissolved by adding KCl to a final concentration of 0.5 *M*. The material so obtained was identified as actomyosin by its viscosity, by the decrease of viscosity on addition of ATP, and by the fact that threads pulled from it in 0.05 *M* KCl shortened on addition of ATP.

In some instances controlled dialysis in the cold was used instead of the usual dilution to precipitate the myosin. This modification permits estimation of the amount of water soluble protein. The residue may be extracted with 0.05 N NaOH in order to determine the amount of noncollageneous nitrogen. It appears that the myosin fraction is diminished in dystrophy. This decrease is the more remarkable since it was observed on dystrophy muscles where other parameters (water content, sodium, and potassium concentration) showed relatively small changes from the normal.

The material obtained by this procedure from normal human or goat muscle appeared to be a fairly homogenous preparation of myosin when studied in the ultracentrifuge at low temperature. The same procedure applied to human dystrophy muscle yielded polydisperse material. This finding was thought to be in general agreement with the several different molecular lengths found in myosin prepared from myopathic human muscles.[2]

It was found that myosin from vitamin E deficient rabbits precipitates at higher salt concentrations than myosin prepared from normal muscle.[3] Dialysis of the KI extract of human dystrophy muscles against 0.2 *M* KCl (buffered to pH 7 with sodium phosphate) precipitated about half of the myosin, with the other half precipitating at 0.1 *M* KCl. This new step introduced into the preparative procedure resolved the polydispersity of the dystrophic myosin into better defined components.

For the individual characterization of these components it was thought necessary to work out preparative details applicable to the limited amount of material available. In the search for more abundant dystrophy material on which different preparative procedures could be tested, extracts of mouse muscle have been studied. These mice came from the strain in which a condition closely resembling human dystrophy is inherited according to a recessive scheme.[4] The mice were sacrificed after definite signs of their abnormality were established in the form of persistently low or declining weight as compared to normal littermates or in the form of seeing "clinical" signs of the disease. The human dystrophy muscles studied represented early stages of the disease. Therefore, the mice were sacrificed not later than seven to ten days after the first noticeable signs.

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Potassium iodide (KI) extracts of the muscles of mice showing "clinical" signs of the disease gave patterns in the ultracentrifuge similar to those seen in the case of the human dystrophy material, that is, the myosin fraction appeared to be polydisperse. In contrast to the findings on normal human and goat muscles, however, normal mouse muscles also gave polydisperse myosin when extracted with KI. When KCl was used for extraction, the myosin fractions of both normal and dystrophic mouse muscles again appeared to be similar in the ultracentrifuge, and all preparations seemed to be somewhat less polydisperse.

There was an apparent decrease of the myosin fraction in extracts of mouse dystrophy muscle relative to other muscle proteins; this is similar to the observation on human muscle. The possible significance of these findings is discussed.

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A collagen-like compound isolated from spinal cord

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IN THE SEARCH for the allergic encephalomyelitic agent known to be present in nerve tissue, bovine spinal cord was fractionated and the various fractions tested for activity in the guinea pig. It was found that the lipid-free residue has a higher activity than the organic solvent soluble fractions. It was further found that the activity of the lipid-free residue is not attenuated in comparison to the starting material,[1] as previously reported for other preparations.[2]

EXPERIMENTAL

The lipid-free residue was extracted with water by autoclaving at 15 pounds pressure, and the extract was dialyzed and lyophilized. It was found that about one-fifth of the extracted material was removed by dialysis. Preliminary tests disclosed that the nondialyzable fraction is protein in nature, with a small amount of glycogen and nucleic acid, the latter indicated by the presence of ribose on a chromatogram after hydrolysis. Ammonium sulfate fractionation yielded four fractions. The most active fraction, which accounted for about 40 per cent of the total weight, was obtained at 60 per cent saturation. This was further purified and its physicochemical properties studied.

PROPERTIES OF THE COMPOUND

Ultracentrifugal and electrophoretic measurements indicated that this protein consists of one major component.** The compound contains 17.61 per cent nitrogen (by Dumas) but no sulfur or phosphorus. Chromatography of the hydrolysate[3] revealed the presence of hydroxyproline and hydroxylysine, characteristic components of collagen. The only protein from mammalian tissue which contains a significant amount of hydroxyproline is collagen. Therefore, it was considered that the compound belongs to the collagen group. Quantitative analysis of all the amino acids has been carried out by the use of microbiologic methods,[4] except hydroxyproline and hydroxylysine. These two amino acids were determined by other procedures.[5,6]

The amino acid composition of this protein was compared with that of collagen obtained from various sources. The values are very close, with the possible exception of glycine, which is somewhat lower (20.3 per cent) in our compound; collagen contains about 26 per cent. Recently a fibrous protein was isolated from vitreous humor of the eye, called vitrosin by the authors,[7] and, although atypical, it was classified as a member of the collagen class. Vitrosin contains 19 per cent glycine. Detailed comparative studies would be necessary to establish whether our protein has a closer relationship to vitrosin than to the more conventional type of collagen.

The carbohydrate content was determined quantitatively and the constituent sugars identified by paper chromatography. Glucose, mannose, galactose, and fucose were found. The presence of glucose, mannose, and galactose has been demonstrated in collagen.[8] The minimum molecular weight calculated from the amino acid analyses was found to be 39,077. Bowes and Kenten[9] reported a minimum molecular weight for collagen of 38,730 calculated by the same procedure.

**The results of these measurements, including molecular weight from sedimentation constants, will be published elsewhere by Dr. M. Mackenzie.

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ALLERGIC ENCEPHALOMYELITIC ACTIVITY

This compound, when injected into guinea pigs, induces encephalomyelitis. It has been compared quantitatively with lyophilized bovine cord and found to have a specific activity of two to two and one-half times that of the original cord. In view of the yield of this compound (3.2 per cent of cord acetone powder) and its state of purity, this enrichment in specific activity is not sufficient to warrant the conclusion that it is the principal active constituent of bovine cord. Another protein with higher specific activity has been obtained from the same starting material. Whether the activity of the collagen-like protein is an intrinsic characteristic or results from traces of the more active antigen cannot be stated at present. It would be interesting to prepare collagen from some other tissue in the same way as this protein, and to test it for allergic encephalomyelitic activity under the same conditions.

SUMMARY

A homogeneous fibrous protein has been isolated in a high state of purity from bovine spinal cord and its physicochemical properties studied. Complete quantitative amino acid analyses have been made. Based on the high hydroxyproline content and the presence of hydroxylysine, it is suggested that this protein belongs to the collagen group.

The compound induces allergic encephalomyelitis in guinea pigs, and its specific activity is greater than that of the crude cord material from which it was prepared. The increase in activity, however, is not sufficient to warrant its designation as the active agent, but rather suggests the possibility that a very active fragment, itself a protein or polypeptide, is adsorbed on the collagen.

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Nucleic acid content of cerebral neurons

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NUCLEIC ACID ANALYSES are being performed as part of an investigation of metabolic correlates of neuronal activity under various conditions. The initial analyses have been made on various areas of the rat brain to establish a reproducible analytic scheme and provide a base line for any variations that might be observed when the brain had been subjected to various physiologic stresses or agents. Although the many regions of the brain differ markedly from one another in metabolic as well as structural ways, prior nucleic acid analyses for the most part have been performed on the whole brain, ignoring the internal differences. Perhaps this is the factor that is at least partially responsible for the marked variability to be found in the literature for RNA and DNA values on whole brain (for DNA in terms of DNA-P/100 gm., 13.5-36.8 mg.; for RNA in terms of RNA-P/100 gm. tissue, 9.4-19.1 mg.; for the RNA/DNA ratio 0.94-3.1).*

Although many of the procedures previously employed by other investigators were tried, several were found to be inadequate. The procedure finally adopted was the ultraviolet method of Logan and co-workers.[1] Two pairs of duplicate samples were run to determine the total nucleic acid and DNA. The Schneider extract of residue from lipid extraction (5 per cent TCA, hydrolyzed for 15 minutes at 90° C.) was read at 268 mμ when total nucleic acid was measured. The Schmidt-Thannhauser procedure was followed in the determination of DNA. The lipid residue was extracted overnight at 37° C. with a volume of 1 N potassium hydroxide equal to ten times the weight of the tissue being analyzed. A known aliquot of the alkaline extract was acidified with 0.2 the volume of the aliquot of 6 N hydrochloric acid containing 5 per cent TCA. The residue was extracted in the same manner as the Schneider extract of total nucleic acid. The ultraviolet absorbance was read at 268 mμ. An attempt was made to use the perchloric acid method developed by Ogur and Rosen for plant tissue and modified for mammalian tissue by Moule. This procedure gave elevated values for DNA due to interfering ultraviolet contaminants, which presumably are protein degradation products. Logan and co-workers reported similar results.

It had been observed that TCA solutions of both DNA and RNA which had been hydrolyzed for the same length of time have the same absorptivity at 268 mμ. The absorptivity which is used here is stated in terms of mg. NAP/cc. It is readily converted to the atomic extinction coefficient by multiplying by 30.98. Several factors are involved in the use of this crossover point. First, the value for a_p has been found by us (as well as by others) to be 320 for RNA, using commercial preparations. The correct a_p awaits measurements on nucleic acid from neural tissue. A second factor concerns the following of Beers' law when dealing with concentrations less than 45 DNA/cc. or 4.0 DNA-P/cc. Previous reports by Blout and Asadourian would support such agreement.

The effect of preliminary treatment in the selection of the tissue is important. The head is separated from the animal following a stunning blow on the head. The brain is removed and the different areas separated while the tissue is kept cold. The various parts are placed in ice water to remove surface blood and are then weighed and analyzed. It has been found that more reproducible results are obtained if the tissue pieces are blotted prior

*Abbreviations: RNA = ribonucleic acid; DNA = deoxyribonucleic acid; P = phosphorus; TCA = trichloracetic acid; NAP = nucleic acid phosphorus; TNA = total nucleic acid.

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TABLE 1
NUCLEIC ACID CONCENTRATIONS IN THE DIFFERENT AREAS OF THE RAT BRAIN, IN MG. P/100 GM. WET WEIGHT TISSUE

Area	*Preparation*	*TNA*	*DNA*	*RNA*	*RNA/DNA*
Cerebral cortex	Frozen (unblotted)	21.2 ± 7.1	6.9 ± 1.0	14.3 ± 7.2	2.1 ± 1.1
	Frozen (blotted)	22.1 ± 2.1	7.5 ± 0.1	15.6 ± 2.3	2.0 ± 0.3
	Fresh	22.5 ± 1.3	7.0 ± 0.6	15.5 ± 1.5	2.1 ± 0.3
White matter	Frozen	17.0 ± 1.4	8.8 ± 2.3	8.2 ± 2.7	0.93 ± 0.4
	Fresh	18.4 ± 2.7	8.4 ± 1.1	10.0 ± 2.9	1.2 ± 0.4
Cerebellum	Frozen	50.0 ± 1.5	39.4 ± 4.5	10.6 ± 4.7	0.27 ± 0.12
(whole)	Fresh	58.3 ± 2.8	38.2 ± 4.0	20.1 ± 4.9	0.53 ± 0.14
Medulla	Frozen	13.9 ± 2.3			
	Fresh	13.0 ± 1.7			

to weighing (table 1). A comparison of cerebral areas which had been kept frozen with fresh tissue samples shows that the total nucleic acid in the cerebellum is decreased in the frozen tissue. This difference in the cerebral cortex and white matter is statistically not significant. The DNA content is the same in the fresh and frozen tissues of the cerebral cortex and cerebellum. The RNA in the cerebellum is reduced by approximately 44 per cent in frozen cerebellum, while the DNA is the same in both preparations. The total nucleic acid in these three areas, when allowed to remain warm for about five minutes, is decreased by 20 per cent in the cortex, 27 per cent in the white matter, and 37 per cent in the cerebellum. This decrease in the cerebellum is greater than the RNA content so that DNA must also be degraded.

There are several hypotheses which could be advanced to explain these differences: 1) A particularly labile RNA is present in the cerebellum which is degraded during the slow freezing and thawing process, or it is present in greater amounts in the cerebellum than the cortex. 2) An enzyme is present to a larger extent in the cerebellum than in the cerebral cortex, which degrades the RNA during the freezing and thawing process. It is known that different enzymes are present in the different areas of the brain, and Jordan has obtained evidence that different amounts of ribonucleases are to be found.

To test this last hypothesis, homogenates of cerebral cortex and cerebellum were assayed for the ribonuclease and 5′-nucleotidase activity. There appears to be more ribonuclease activity and less 5′-nucleotidase in the cerebral cortex than in the cerebellum. Hence the ribonucleic acid in the cerebellum may be degraded more rapidly because of the larger concentration of the 5′-nucleotidase.

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Quantitative histochemistry of selected central tracts

D. B. McDougal, Jr., M.D.*

THIS IS PART of an attempt to correlate chemical composition with structure and function within the nervous system. Central fiber tracts offer real advantages for this purpose, because of their comparative simplicity. Nevertheless, a list of anatomic and physiologic characteristics which might well be expected to influence the chemical structure of a tract would include the following: axon caliber, length of axon, amount and type of myelin, amount and type of glia, distance of the sample from the cells of origin of the tract, vascularity, and function. Since many of these characteristics are unknown or are known only very imprecisely for any tract, only fiber caliber and degree of myelination, which tend to vary together, have been considered in the present study.

METHODS

Tissues were removed from the animal (rabbit) as soon after decapitation as possible and frozen in liquid nitrogen. Methods of handling, dissecting, and weighing the tissue have been described by Lowry.[1] Cross-sections of the tracts were cut at 25 micra and dried at −40° C. Pieces used for assay included the whole cross-section of the tract or anatomically specified portions of the cross-section; these weighed from 0.5 to 3 micrograms. The pyramidal tract (at the level of the medulla) was divided into medial and lateral halves. Dorsal columns (midcervical cord) were divided into dorsal, middle, and ventral portions. The optic tract was divided into six pieces from ventrocaudal to dorsorostral across the long axis of the tract. The most ventrocaudal piece probably corresponds to the small-fiber portion of the optic tract, as described for the cat by Bishop and Clare[2] and Bishop and associates.[3]

Total lipid and fat-free dry weight were determined gravimetrically.[1] The method of Albers and Lowry[4] for total cholesterol was modified so that cholesterol was extracted directly with the trichloroethane-acetic anhydride mixture for 20 minutes at room temperature, and fluorescence was developed in the presence of the tissue.

Fumarase (fum.) and glutamic-aspartic transaminase (trans.) were measured by fluorometric methods.[5,6] Phosphoglucoisomerase (isom.) was measured colorimetrically.** Glucose-6-phosphate dehydrogenase (G6PDH)[7] and isocitric dehydrogenase (ICDH)† were determined by fluorometric measurement of the TPNH produced.

Total extractable lipid is reported as per cent of dry weight, cholesterol as moles per kilo total lipid, and enzymes as moles of substrate acted upon or product produced per kilo fat-free dry weight per hour.

RESULTS AND DISCUSSION

The tracts used were selected to give a wide range of average fiber calibers. Fornix, habenulo-interpeduncular tract, mammillothalamic tract, and the bundle of fibers lying along the caudal and ventral aspect of the optic tract are composed largely of very small fibers; dorsal columns contain many large fibers; and the rest of the optic tract, olfactory tract, and pyramidal tract fall in between these two extremes. On the basis of lipid content, these tracts fall into three distinct and widely separated groups, the small-fiber group being relatively poor in lipid, the large-fiber group high

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TABLE 1

Tract	Total Lipid[1]	Cholesterol[2]	Isom.[3]	G6PDH[3]	ICDH[3]	Fum.[3]	Trans.[3]
Dorsal columns 1	76.1	.657	95	6.34	4.70	9.30	6.45
Olfactory	69.9	.561	123	2.53	3.86	14.1	9.54
Mammillothalamic	57.8	.526	117	1.44	5.79	29.8	24.0
Optic 1	61.5	.572	226	2.34	4.96	8.15	22.6
5 & 6	71.7	.596	310	2.05	2.48	5.76	11.1
Average brain	30	.68	250	1.50	8	40	60

[1]As per cent of dry weight. [2]Moles per kilo total lipid. [3]Moles per kilo fat-free dry weight per hour.

in lipid, and the tracts with intermediate fiber caliber also being intermediate in lipid content. Cholesterol distribution followed total lipid distribution fairly closely. Table 1 gives an example from each group, plus the small-fiber portion (optic 1) and a sample from the rest of the optic tract (optic 5 and 6), which belong to the small-fiber and intermediate-fiber groups, respectively, but vary somewhat from other members of their groups in enzyme activity.

Large differences in enzyme activities were found among the tracts. Phosphoglucoisomerase was present at about the same levels in representatives of all three groups, but was high in optic tract. High levels of some glycolytic enzymes, including isomerase, have been reported previously for this tract.[8,9] In contrast, optic tract was lower in fumarase than other members of the corresponding groups. With this exception, the tracts showed increasing fumarase activity with decreasing lipid content. Transaminase also varied from group to group in this way, with optic tract no exception. Glucose-6-phosphate dehydrogenase, on the other hand, was present at higher levels in tracts with high lipid content than in those with low lipid content. Isocitric dehydrogenase activity was somewhat lower in the intermediate group than in the other two.

Interpretation of these data in terms of tract structure and function is difficult and premature, in view of the lack of precise anatomic information. The presence in optic tract of high activities of some glycolytic enzymes, combined with low fumarase activity, might suggest that optic tract is more dependent on glycolysis for its energy than the other tracts. If so, whether this is related to differences in blood supply, rate of energy consumption, or some other factor cannot be determined at present. It does not seem unreasonable that, in tracts with low lipid content and hence a high concentration of protein, a high concentration of transaminase should be found. However, there does not seem to be any reasonable explanation for the association of a high glucose-6-phosphate dehydrogenase activity with either a high lipid or low protein content or, for that matter, with large caliber or heavily myelinated axons. Nevertheless, it is believed that the chemical differences are reflections of structural and functional differences among the several tracts, and that eventually it will be possible to discuss the chemical structure of a tract in terms of its anatomic components and physiologic attributes.

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Substrate utilization of peripheral nerves

I. H. Heller, M.D. and S. Hesse, M.D.

RECENT clinical interest in the peripheral neuropathies has prompted the present study of substrate utilization of peripheral nerves. As is well known, demyelinating lesions of nerves can be caused by a wide variety of etiologic agents and conditions, resulting in fairly similar clinical disturbances. The mechanism by which interference with normal function occurs is not known.

The multiple types of peripheral neuropathy suggested to us that the interference with metabolism in each case occurred at a common level or levels of intermediate metabolism. The present study was designed to serve as a background for the investigation of the metabolic defects in different forms of demyelinating diseases of peripheral nerve. We have tried to establish a pattern of substrate utilization by nerves from normal animals in order to compare it with the patterns obtained from pathologic material. Studies during certain physiologic states have also been performed.

METHODS

Sciatic nerves were obtained from adult Wistar rats. Normal rats were killed by a blow on the head and then decapitated. Blood was collected for determination of glucose. Normal values were 120 mg. per cent. Respiration was measured in the standard manner as follows. Each nerve was placed in a Warburg flask containing phosphate-buffered medium resembling spinal fluid. The initial pH of the medium was 7.8 to 8.0. Various substrates were added at the start of the experiment to one of the vessels to give a final concentration of 0.01 *M*; the other vessel containing the other member of the pair of nerves served as a substrate-free control.

A further series of similar studies was performed on rats which were fasted for 24 hours and then given 25 units of crystalline zinc insulin intraperitoneally. After three hours the animals were comatose and were then killed, as described above. Blood sugar levels were consistently below 40 mg. per cent in all comatose animals.

RESULTS

Autoxidation

The rate of oxygen uptake for normal nerves, without substrate addition, varies considerably from animal to animal. The autoxidation rate of nerves from insulinized animals was usually depressed and showed less scatter. In both instances the rates decreased with time, and differences between the means were statistically significant even during the third hour.

Effects of Added Substrates

Glucose, mannose, fructose, sucrose, pyruvate, and succinate all produced a definite increase in oxygen uptake in nerves from both normal and insulinized animals. The effect of glutamic acid was less marked. In general, moreover, the rates of nerves from insulinized rats with added substrate approach those of normal animals with the corresponding substrate.

Glycine, fumarate, and galactose had no apparent effect on respiration of nerves from normal animals. The preparations from insulinized animals showed a more definite response to fumarate and galactose than did the nerves from normal animals; however, the small number of experiments precludes the drawing of definite conclusions on this point.

The addition of insulin to the medium containing glucose or mannose caused no change in the respiratory rate of nerves from insulinized animals.

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The addition of sodium fluoracetate to the medium had no effect on the autoxidation rate of normal nerves, nor did arsenious trioxide.

Following the work of Geiger, who showed enhanced glucose uptake by brain *in situ* in the presence of uridine and cytidine, these substances were added to the medium of normal nerve, both singly and in combination and together with glucose. No appreciable difference in the oxygen uptake rate was noted in any of these preparations.

An attempt was made to render the animals hypoglycemic and comatose by the injection of phlorizin. Although a persistent glycosuria was obtained, the blood sugars were only slightly below normal, around 80 mg. per cent, and no coma was obtained. The autoxidation activity of these nerves was within normal range.

It is planned to induce lesions in peripheral nerves by section of the nerves, by the administration of toxic substances, by dietary deficiency, and by the production of an allergic neuropathy. The results of substrate utilization under these conditions will be studied and compared with those obtained in the normal animal. Possible interference with the metabolic activity of these nerves will be sought.

Increase in rate of glucose utilization during activity in excised sympathetic ganglia

Michel Dolivo, M.D.* and Martin G. Larrabee, Ph.D.

METABOLIC MEASUREMENTS at 37° C. on sympathetic ganglia excised from rats have previously shown that oxygen consumption accelerates when activity is caused by preganglionic nerve stimulation. Consumption of exogenous glucose also accelerates, but the increase is entirely accounted for as lactate produced.[1–3] Therefore, this additional oxygen must combine with endogenous substrates. This agrees with conclusions of other investigators concerning substrate utilization in excised nerve trunks and brain.[4,5]

After the above experiments were performed, it was found that some ganglion preparations have anoxic cores at 37° C. but that none are anoxic at 23° C.[6] Therefore, the observations were repeated at 23°. Effects of pH were also investigated.

Experiments concerning glucose uptake and lactate output were divided into five successive periods: 1) an initial resting period, two hours in length; 2) a period of one-half hour during which the preganglionic nerve was stimulated supramaximally at an average frequency of 4.6/sec.; 3) a period of two hours during which the stimulation was continued; 4) a recovery period of one-half hour immediately after stopping stimulation; and 5) a final resting period of two hours. Resting rates of glucose uptake and lactate output corresponding to periods 2, 3, and 4 were determined by interpolation between periods 1 and 5. Excess amounts in periods 2, 3, and 4 above the interpolated resting rates were considered to be activity increments, and their sum was divided by 2½ to obtain the increment in activity rate per hour of stimulation. Results are given in the accompanying table.

The following conclusions may be drawn concerning metabolism at 23°:

1. Glucose uptake and lactate output were both accelerated by activity. It may be emphasized that these increases could not be due to a Pasteur effect operating in an anoxic core, as had been thought possible in interpreting the earlier experiments at higher temperature.

2. Both the resting output of lactate and the activity increment in output of lactate declined significantly as the pH was lowered.

3. The mean difference between the activity increments in glucose uptake and lactate output, which is a measure of the activity increment in glucose available for oxidation, increased as the pH was lowered. This pH effect was of doubtful significance statistically.

4. With the neurons at rest, the rate of oxygen uptake was in good agreement with the rate at which glucose was available for oxidation at all values of pH.

5. At pH 7.0 and 6.8 the activity increment in oxygen uptake was in good agreement with the activity increment in glucose available for oxidation. The agreement was poor at pH 7.4, although even here the disagreement was not statistically significant.

Thus, in general, it may be concluded that oxygen uptake is adequately accounted for by glucose metabolism at 23° C. in active ganglia, as well as in resting ganglia. This contrasts with previous findings at 37° C. where endogenous substrate must have been oxidized during activity.

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*Fellow of the Swiss Academy of Medicine, 1955-1957.

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TABLE 1

METABOLIC RATES AT 23° C. — MG./G. DRY WT./HR. (Mean ± s.e.m.)

	pH:	7.4	7.0	6.8
	No. of Observations:	8	7	6
1. Glucose uptake	At rest	4.5 ± 0.2	4.3 ± 0.3	3.7 ± 0.4
	Activity increment[1]	4.6 ± 0.4	4.3 ± 0.4	3.4 ± 0.4
2. Lactate output	At rest	1.1 ± 0.1	0.9 ± 0.1	0.6 ± 0.1
	Activity increment	3.8 ± 0.4	2.7 ± 0.4	1.4 ± 0.2
3. Glucose uptake minus lactate output	At rest	3.4 ± 0.2	3.4 ± 0.3	3.1 ± 0.4
	Activity increment	0.8 ± 0.4	1.6 ± 0.5	2.0 ± 0.4
4. Oxygen uptake as glucose equivalent	At rest[2]		3.3 ± 0.2	
	Activity increment[3]		1.7 ± 0.4	

[1]Activity increments in glucose and lactate exchanges = total excess caused by 2½ hours of stimulation at 4.6 per sec. divided by 2½.

[2]Mean of ten observations (approximately independent of pH).

[3](Mean resting rate) × (mean of six determinations of peak percentage increase during stimulation at 4.6 per sec.) × (0.88). The latter factor was estimated to allow for decline from the peak rate during 2½ hours of stimulation (Percentage increase was independent of pH).

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Effect of glutamate and glutamine on the intracellular electrolytes of nervous tissue

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In the course of an investigation of factors controlling the transfer of water and electrolytes across nerve cell membranes, we have examined the effect of different external concentrations of glutamate and glutamine on the volume and electrolyte composition of the intracellular fluid of rabbit retinas.

Retina was selected as the tissue in which to study these phenomena because of characteristics making it unusually suited for study *in vitro*. About one-eighth of the retina is specialized for photoreception. The rest appears, on the basis of its embryology, morphology, function, and chemistry, to be basically similar to other examples of central nervous system gray matter. The retina offers four important advantages: it can be removed rapidly without being cut; it is thin enough (0.1 mm.) to be nurtured by diffusion; it is tough enough to remain intact during incubation; and it is of sufficient mass to permit multiple analyses by semimicro methods.

The experiments consisted of isolating the retinas from anesthetized rabbits in a warm room at 98° F. and analyzing them, either immediately or after incubation in a control medium or a test medium. The retinas were analyzed for wet weight, dry weight, total water by difference, sodium, potassium, chlorides, and inulin. Recovery experiments showed that the analytic error could be expected to average not more than 1 per cent. The amounts of water and electrolytes that were intracellular were estimated by subtracting from the total water and electrolytes those calculated to be extracellular, using inulin to define the volume of the extracellular space.

The control medium corresponded to spinal fluid in electrolyte composition and contained a bicarbonate buffer and glucose as substrate. It was equilibrated with a gas phase of 5 per cent carbon dioxide and 95 per cent oxygen. Sodium L-glutamate, sodium D-glutamate, or L-glutamine were added as solids to the basic medium.

Retinas incubated for up to one hour in the control medium appeared, on histologic examination, to be indistinguishable from *in vivo* retinas; and their intracellular water and electrolytes showed little effect from their having been isolated and maintained *in vitro* (figures 1 and 2).

Retinas incubated with glutamate showed an increase in intracellular water and total monovalent cation (figure 1). An effect was evident with as little as 0.5 mM/L of glutamate. The D-isomer had essentially the same effect as the natural compound. These changes were probably the result of an active accumulation of glutamate by the incubating cells, the glutamate being accompanied by an equivalent amount of cation and by sufficient water to maintain isotonicity. The amount of cations and water entering the cells was quantitatively in accord with the amount of glutamate found by Terner and associates[1] to accumulate in ox retinas incubated in comparable external concentrations of glutamate.

When the movements of the individual ions were examined in these same retinas (figure 2), the effect of the glutamate appeared more complicated. Changes were again evident with as little as 0.5 mM/L and much more marked with 5 mM/L. Potassium left the cell and sodium and chlorides entered during the first ten minutes, followed by a rather abrupt

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This study appears in detail in J. Neurophysiol. 19:201, 1956.

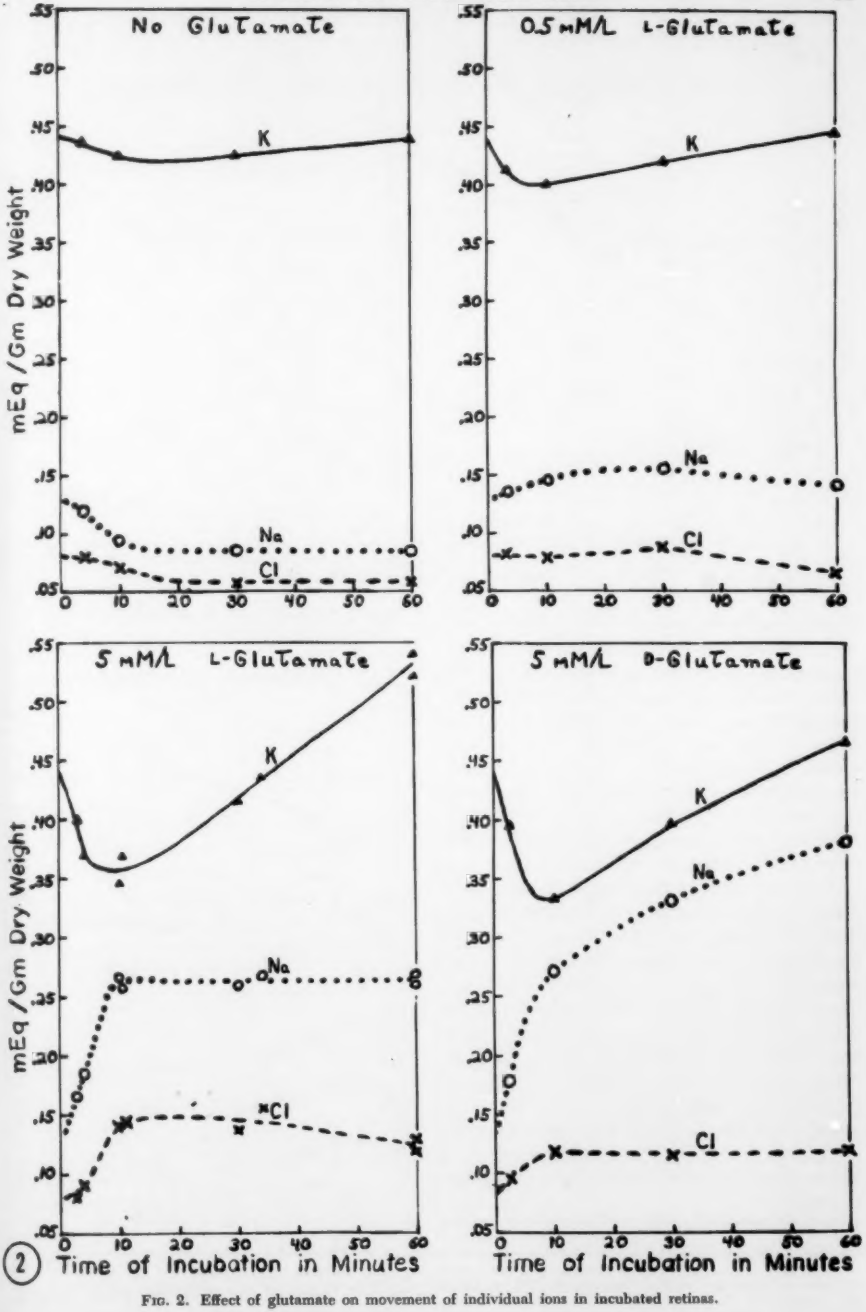
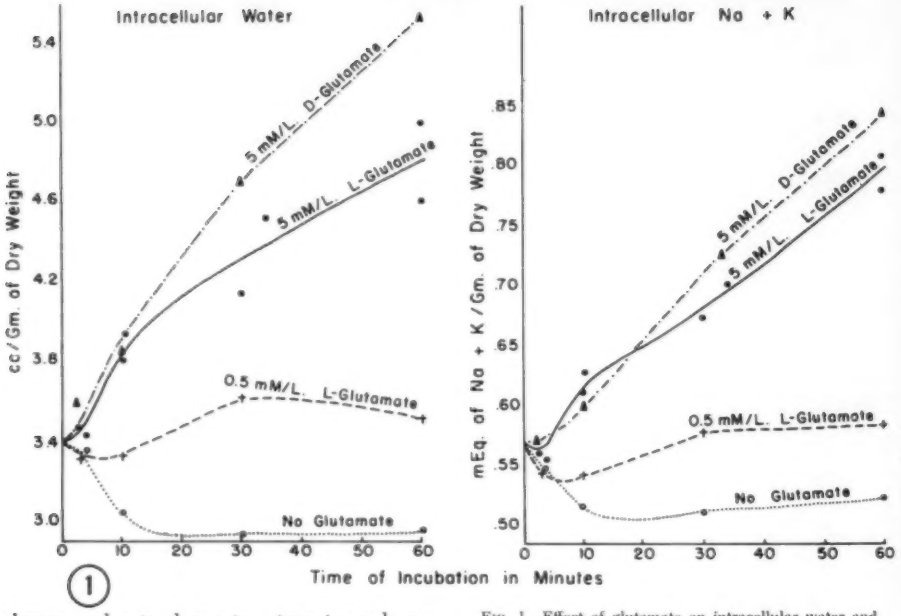


FIG. 2. Effect of glutamate on movement of individual ions in incubated retinas.



change and a tendency to return toward normal. The medium was presumably not changing during this period, as its volume was 400 times that of the tissue. The events in the first ten minutes may have been related to a rapid influx of glutamate following its sudden increase in the extracellular fluid; and the subsequent return of the electrolytes toward normal may have coincided with the reestablishment of a more normal concentration gradient of glutamate across the cell membrane.

The changes occurring during incubation in 5 mM/L of L-glutamine are shown in figure 3. The intracellular water steadily increased and there was a concomitant dilution of the intracellular potassium, sodium, and chlorides. These changes were probably secondary to the active accumulation of the osmotically active but uncharged glutamine molecule. The increase in intracellular glutamine required to produce these effects can be calculated to be about 33 mOsm/L, which is of the same order of magnitude as the increase of glutamine in brain slices incubated under comparable conditions.[2]

FIG. 1. Effect of glutamate on intracellular water and cations of incubated retina.

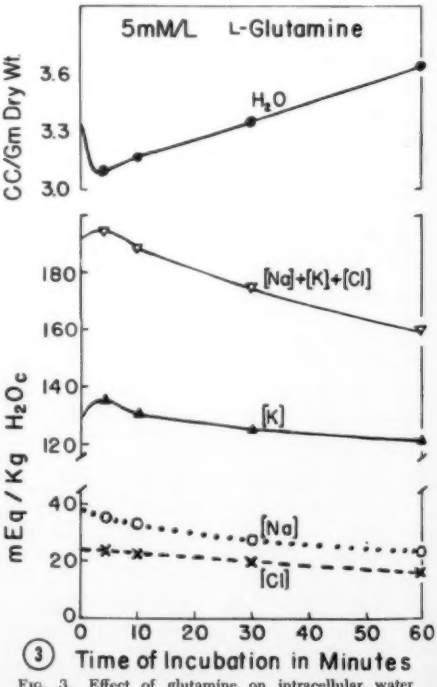


FIG. 3. Effect of glutamine on intracellular water and ions during incubation of retinas.

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Diffusion constants in the extracellular space of brain tissue

Hugh McLennan, Ph.D.

KINETIC STUDIES of the movements of ions and molecules across the cellular boundaries of organized animal tissues require a knowledge of the effective diffusion constants holding in the extracellular spaces. A direct attempt to measure these diffusion constants has been the aim of the work reported in this paper.

Slices of the cerebral cortex of rats and/or of their livers were placed on small silver screens and incubated in appropriate saline media. The screens ensured that the slices remained flat during the course of the experiment, and the degree of swelling during the incubation was estimated by weighing the slices before and after immersion. Radioactive isotopes of sodium and potassium were added to the media where the diffusion of these substances was being followed. The rate of uptake of inulin and sucrose from media to which these substances had been added was determined by chemical analysis of the slices after various times of incubation. Full details of the experimental methods have been given elsewhere.[1,2]

The equation governing diffusion into a plane sheet is given by equation 1 (figure 1), where S is the concentration of tracer substance and D the diffusion constant. Integrating and inserting the appropriate boundary conditions gives equation 2 where the thickness of the sheet is given by 2b.

$$\frac{dS}{dt} = \frac{D}{\lambda^2} \cdot \frac{d^2S}{dx^2} \qquad (1)$$

$$\frac{\bar{S}}{S_o} = 1 - \frac{8}{\pi^2}\left[e^{-\frac{D\pi^2 t}{4b^2\lambda^2}} + \frac{1}{9}e^{-\frac{9D\pi^2 t}{4b^2\lambda^2}} + \cdots\right] \qquad (2)$$

$$\lambda = 1 + 3\frac{Vc}{Vt}\left(1 - \frac{\pi}{4}\right) \qquad (3)$$

FIG. 1. Equations for calculating the diffusion constant, D. See text for details.

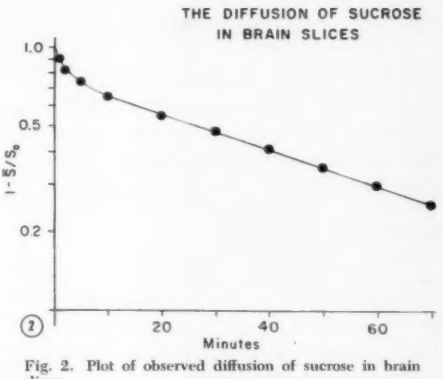

Fig. 2. Plot of observed diffusion of sucrose in brain slices.

In these equations λ is a factor by which the distance that a particle penetrating into the sheet has to travel is increased by the presence of impenetrable obstacles, that is, the tissue cells. It can be shown[2] that for the presence of spherical cells, which both brain and liver cells have been assumed to be, λ is given by equation 3, where Vc and Vt are the volumes of cells and of the whole sheet, respectively.

Using these equations, the experimental curves obtained (as in figure 2) can be analyzed and values for the diffusion constant D found. The results obtained in the present study are summarized in table 1, where the diffusion constants for various substances in the extracellular spaces of brain and liver are shown and contrasted with the values for free solution. It can be seen that, for sodium ions and for inulin and sucrose, the diffusion constants are of the same order of magnitude as those holding in free solution; however, that for potassium ions is much reduced in

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TABLE 1
DIFFUSION CONSTANTS AT 20° C.

		Measured Extracellular D, $cm^2/min.$	*Free Solution D, $cm^2/min.$*
Brain	K+	5.6×10^{-5}	9.9×10^{-4}
	Na+	8.0×10^{-4}	7.2×10^{-4}
	Sucrose	2.1×10^{-4}	3.1×10^{-4}
	Inulin	1.9×10^{-4}	1.0×10^{-4}
Liver	K+	3.7×10^{-4}	9.9×10^{-4}
	Na+	9.1×10^{-4}	7.2×10^{-4}

brain. This slow extracellular diffusion of potassium has been found also in muscle; however, it is not apparent in liver (table 1). The slow diffusion, therefore, appears to be a property of excitable tissues, and further to be specific for potassium. It seems possible that what is happening here is an adsorption of the substance onto the surfaces of the cells.

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Mitochondria in brain as the sites of its metabolic activity

H. G. du Buy, Ph.D.

PRESENT KNOWLEDGE concerning mitochondria suggests that a better understanding of the functioning of the brain, and of the effects of pathogenic and chemical agents on it, can be obtained by studying its mitochondrial fraction. A comparison of staining and destaining phenomena with Janus green B of mitochondria in living cells and of centrifugally fractionated mitochondria[1] indicates that brain mitochondria withstand isolation procedures better than mitochondria from other sources, such as liver. Thus, data obtained with isolated brain mitochondria are considered to apply more nearly to mitochondria of intact cells than those obtained with isolated mitochondria from other organs.

Isolated brain mitochondria have been shown to carry a number of functional enzyme systems and enzyme complexes. Typical reactions essential in protein metabolism, carried out by brain mitochondria, are: oxidation of glutamate and inorganic phosphate uptake with glutamate as substrate,[2] and the ability to split the peptide bond of glycylglycine. An example of fatty acid metabolism is the oxidation of β-hydroxybutyrate. An example of ribotide metabolism is the oxidation of uridylic acid. Brain mitochondria in addition carry the following carbohydrate utilizing enzyme systems: glycogen, glucose, fructose, mannose, fructose-1-P (Hers) and the hexose monophosphate shunt systems.[3] The mitochondria also carry the Krebs enzyme cycle[4] and the aerobic oxidase system (cytochrome system, dopa-oxidation over this system). Because they carry the Krebs enzyme cycle and terminal oxidases, the mitochondria use oxygen, as well as accumulated lactic acid, when carbohydrates are glycolysed. Brain supernatants, by contrast, glycolyse as do the mitochondria, but have no functional oxidase system. Hence, they do not metabolize carbohydrates beyond lactic acid, and they glycolyse independently of the presence of oxygen.

The extent to which incomplete enzyme sequences occur in supernatants has been studied by substituting hydrogen-transferring dyes for the oxidases. Thus, Janus green B ($10^{-4}M$) was used to substitute for the cytochrome system, and 5-methyl-5-phenazinium methylsulfate ($2 \times 10^{-3}M$)[5] was used to substitute for the flavo-protein and the cytochrome system. This work, summarized in table 1, shows that brain supernatants have fewer complete enzyme complexes than liver supernatants. In contrast, brain mitochondria have been shown[3] to carry more complete multienzyme systems than liver mitochondria. In conjunction with data on staining and chemical composition, results constitute further evidence for the relative stability of brain mitochondria during isolation, and support the view, expressed previously,[3] that "all enzyme systems necessary for maintenance of the cell can be expected to be on all mitochondria."

In regard to this view, the data of table 2 are of interest. They show that when the ratio $\frac{\text{glycolysis}}{\text{respiration}}$ of brain mitochondria is compared with that of slices and the other subcellular fractions, only the mitochondria showed a residual lactic acid level low enough to approach the value for normal tissue. Adding supernatant back to mitochondria, high ratios, similar to those obtained with slices and homogenates, are obtained. Such "glycolytic excess" is considered typical for cancerous tissues. However, such high ratios might well be an expression of the degree to which the glycolytic system is removed from the mitochondria by tissue damage (medium used,

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TABLE 1

FUNCTIONAL LINKAGE OF SOME ENZYME COMPONENTS IN MOUSE SUPERNATANT FRACTIONS*

LIVER	*BRAIN*
1. Aconitase	———
2. Isocitric dehydrogenase—{ DPN / TPN }—flavin	Isocitric dehydrogenase—{ DPN / TPN }———
3. Fumarase	Fumarase
4. Malic dehydrogenase—DPN—flavin	Malic dehydrogenase—DPN———
5. Oxalacetic decarboxylase	Oxalacetic decarboxylase
6. Lactic dehydrogenase—DPN—flavin	Lactic dehydrogenase—DPN———
7. 3-PG dehydrogenase—DPN	3-PG dehydrogenase—DPN
Glycolytic enzyme system from aldolase to lactic dehydrogenase—DPN	Glycolytic enzyme system from hexokinase to lactic dehydrogenase—DPN

*Abbreviations as follows: DPN = diphosphopyridine nucleotide; TPN = triphosphopyridine nucleotide; 3-PG = 3-phosphoglyceraldehyde.

slicing, homogenization).[4,6] Similarly, increase of lactic acid formation following virus infection of tissue culture has been found.[7] It seems that a high ratio $\frac{\text{glycolysis}}{\text{respiration}}$ might well be indicative of the degree to which the glycolytic system becomes soluble under adverse conditions, rather than being indicative only of a cancerous state.

Further, the effect of 2, 4-dinitrophenol (DNP) on lactic acid accumulation and oxygen uptake by mitochondria with glucose or fructose diphosphate (FDP) as substrate is of interest (table 3). (Oxidative phosphorylation by brain mitochondria with Krebs cycle substrates has been reported before.)[2] With ADP, ATP, or both, no clear effect of 10^{-5} *M* DNP on oxygen uptake was found, but lactate accumulation decreased in the presence of glucose and DNP, when compared with the control, whereas lactate accumulation increased with FDP and DNP. Apparently, DNP, an "uncoupler" of oxidative phosphorylation, affects glycolysis only when the glycolytic enzymes are closely associated with the mitochondria, again an indication of integration of glycolysis and respiration in mitochondria. Under similar conditions, DNP had no effect on lactate accumulation by brain supernatants. Malonate (0.04 *M*) inhibited oxygen uptake as well as lactate accumulation by brain mitochondria with either glucose or FDP as substrate, either in the presence or absence of DNP. It did not clearly affect lactate formation by brain supernatant under similar conditions.

Brain mitochondria are not unique in their use of glucose, although liver, spleen, and kidney were found inactive. The tissues listed in table 4 also glucolyse, although at lower rates than brain.

Other properties of brain mitochondria, as their self-perpetuating nature and mutability, can only be inferred by homologizing animal and plant mitochondria.[8,9] The evidence for the function of brain mitochondria as centers

TABLE 2

USE OF GLUCOSE BY SLICES AND FRACTIONS OF MOUSE BRAIN

*Preparation**	*mg. N per Vessel*	*GLUCOSE*	
		$\Delta Q^{air}_{O_2}(N)$ *μ moles*	$\Delta Q^{air}_{lactate}(N)$ *μ moles*
SLICES (50 mg. fresh wt./vessel)	0.61	0.9	15.8
HOMOGENATE (20% by volume)	1.52	1.6	17.4
MITOCHONDRIA (not washed)	0.90	7.1	9.6
SUPERNATANT (not cleared)	0.54	0.9	22.0
MITOCHONDRIA + SUPERNATANT	1.37	2.8	16.7

*Conditions: Glucose 20 *μ* moles/vessel other contents as in [2]. Temp. 38° C. Center well contained KOH. Concentration of fractions: 20% in terms of an assumed 100% concentration of each fraction in undiluted homogenate.

TABLE 3

EFFECT OF DNP ON THE USE OF GLUCOSE OR FDP BY BRAIN MITOCHONDRIA*

	GLUCOSE		FDP	
	$\Delta Q^{air}_{O_2}(N)$ μ moles	$\Delta Q^{air}_{lactate}(N)$ μ moles	$\Delta Q^{air}_{O_2}(N)$ μ moles	$\Delta Q^{air}_{lactate}(N)$ μ moles
ADP —	6.5	11.7	7.1	6.0
DNP (10^{-5} M)	6.7	4.9	6.5	8.9
DNP (10^{-6} M)	6.8	10.6	7.3	6.7
ATP —	5.5	9.7	7.6	5.0
DNP (10^{-5} M)	5.7	7.0	6.2	9.0
DNP (10^{-6} M)	6.5	10.8	6.8	7.0
ADP + ATP —	6.7	11.6	7.2	4.7
DNP (10^{-5} M)	7.8	5.3	6.2	8.3
DNP (10^{-6} M)	6.5	11.9	6.9	7.5

*Abbreviations as follows: DNP = 2,4-dinitrophenol; ADP = adenosine diphosphate; ATP = adenosine triphosphate; FDP = fructose diphosphate.
Per vessel either 8 μ moles ADP or ATP, or 4 μ moles of each. Conditions as in table 2.

TABLE 4

USE OF GLUCOSE BY WASHED MITOCHONDRIA AND CLEARED SUPERNATANTS FROM VARIOUS TISSUES

PREPARATION	FRACTION*	mg. N per VESSEL	GLUCOSE $\Delta Q^{air}_{O_2}(N)$ μ moles	GLUCOSE $\Delta Q^{air}_{lactate}(N)$ μ moles
BRAIN	wM	0.60	4.7	3.5
	S_2	0.09	0.2	22.8
S91 MELANOMA	wM	0.42	3.5	1.6
	S_2	0.87	−0.1	29.4
TESTIS	wM	0.57	1.6	0.3
	S_2	0.57	0.3	1.9
K_2 CARCINOMA	wM	0.51	0.8	0.9
	S_2	1.02	0.1	19.6
HEPATOMA	wM	0.45	0.5	0.4
	S_2	0.75	0.3	35.2

*wM = washed mitochondria; S_2 = supernatant cleared by recentrifugation. Conditions as in table 2.

of metabolic activity enumerated above, together with functions ascribed to them on the basis of homology with other mitochondria, implicate brain mitochondria as the organelles which may have an important role, not only in restoring energy used to propagate impulses, but also in other (e.g. mnemonic) brain functions.

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The adenosine polyphosphates, creatine phosphate and ascorbic acid content of rat brain

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EARLY INVESTIGATIONS have indicated that changes in formation or utilization of cerebral high energy phosphates follow *in vitro* and *in vivo* administration of barbiturates and certain other anesthetic agents.[1–7] Creatine phosphate and adenosine triphosphate have been reported to be elevated in pentobarbital anesthesia in a variety of experimental animals. Conversely, ether anesthesia was unaccompanied by any such alterations.

Careful examination of the literature reveals that these findings generally cannot be validated by statistical analysis. In addition, chemical studies were carried out through barium and calcium fractionation technics which are tedious and often unreliable.

Since cerebral energy-rich phosphates are concerned with energy necessary to maintain nerve impulses, structural integrity, and other cellular metabolic processes, a statistically valid report of alterations in these compounds in states of depression or excitation still appears necessary. The present investigation was undertaken to establish valid levels of creatine phosphate and the adenosine polyphosphates in the normal and anesthetized animal. This was carried out satisfactorily by means of ion exchange chromatography, combined with spectrophotometric analysis. Ascorbic acid was also determined, since we had previously found this compound to have some active role in cerebral phosphorylative processes.[8]

METHODS AND RESULTS

In the initial series of experiments mature rats were sacrificed by dropping them into liquid air. The brain was powdered and dropped into 5 per cent cold perchloric acid. After brief thawing to 0° C., homogenization, and centrifugation, the perchloric acid extract was passed through Dowex-50 ion exchange resin, holding back creatine and allowing creatine phosphate and the adenine polyphosphates to pass through. Creatine and creatine phosphate were then determined by the method of Ennor and Rosenberg.[9] The adenosine polyphosphates were separated by use of Dowex-1 column and measured in the Beckman DU spectrophotometer at 257 millimicrons, according to the method of Siekevitz and Potter.[10] Ascorbic acid was determined by the dinitrophenylhydrazine method of Roe.[11] The results of eight experiments are summarized in table 1. The values for creatine phosphate and adenosine triphosphate were markedly lower than those previously reported in the literature. In addition, anesthesia with ether or pentobarbital produced no significant changes in these compounds.

Despite the reproducibility of these results, failure to obtain high values of creatine phosphate and adenosine triphosphate led to a revision of technic. In the subsequent experiments, weanling rats were sacrificed in the same manner; however, after the frozen brain was powdered, it was immediately homogenized with cold perchloric acid without thawing and continued as described above. Table 2 demonstrates the alteration under these circumstances. The creatine phosphate and adenosine triphosphate levels were approximately three times those observed previously. Pentobarbital anesthesia produced a significant rise in creatine phosphate, unaccompanied by

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TABLE 1

LEVELS OF SOME CONSTITUENTS OF RAT BRAIN FROZEN IN SITU, IN MICROMOLES PER GRAM FRESH BRAIN (MEAN ± S.E.). BRAIN THAWED IN ICE-COLD 5% PERCHLORIC ACID BEFORE EXTRACTION. RATS WEIGHED 165 TO 230 GM.

		Anesthetized	
Constituent	*Control*	*Ether*	*Nembutal*
Ascorbic acid	2.66 ± .08	2.55 ± .09	2.60 ± .09
Creatine phosphate	1.10 ± .20	1.09 ± .15	1.03 ± .16
Creatine	10.8 ± .27	10.7 ± .18	10.6 ± .12
AMP*	.63 ± .10	.51 ± .08	.48 ± .08
ADP	1.36 ± .04	1.37 ± .06	1.37 ± .06
ATP	.79 ± .12	.79 ± .10	.89 ± .12

*Due to poor recovery of AMP from brain extracts going through Dowex 50 column, these values are known to be low.

changes in adenosine triphosphate, adenosine diphosphate, or adenosine monophosphate.

DISCUSSION

Two variations existed between the sets of experiments reported. These were in age of the animal selected and in method of extraction in perchloric acid. Some of our more recent experiments not reported here indicate that age and size of the animal are not involved. Work of other investigators also indicated that brain size alone is of no great significance. Therefore, it appears that the differences observed resulted from changes in the extraction technic. Sufficient break-down of creatine phosphate and adenosine triphosphate followed thawing of the brain to result in artificially low values as well as in failure to demonstrate changes in narcosis. However, powdering rat brain in the frozen state allowed determination of the higher values and demonstration of creatine phosphate elevation in barbituratized animals. No significant changes were found in ascorbic acid levels, although some investigators reported significant increase in the total body ascorbic acid synthesis in pentobarbital anesthesia.[12] Serdyuk[13] reported increased levels of ascorbic acid in the cerebral hemispheres during excitation and decreased levels during narcosis.

CONCLUSION

The *in vivo* levels of adenosine polyphosphates, creatine phosphate, creatine, and ascorbic acid in rat brain have been established by an easily reproducible technic and clear-cut separation of the compounds were obtained. A significant increase in cerebral creatine phosphate levels followed pentobarbital anesthesia, without changes in adenosine triphosphate.

TABLE 2

LEVELS OF SOME CONSTITUENTS OF RAT BRAIN FROZEN IN SITU, IN MICROMOLES PER GRAM FRESH BRAIN (MEAN ± S.E.). BRAIN CRUSHED IN COLD. RATS WEIGHED 40 TO 115 GM.

		Anesthetized	
Constituent	*Control*	*Ether*	*Nembutal*
Ascorbic acid	2.45 ± .12	2.49 ± .15	2.50 ± .12
Creatine phosphate	3.21 ± .05	2.74 ± .51	4.01 ± .14*
Creatine	7.88 ± .21	7.94 ± .45	7.21 ± .22
Total creatine	10.99 ± .21	10.69 ± .13	11.21 ± .27
AMP	.46 ± .06	.42 ± .06	.40 ± .03
ADP	.99 ± .09	.96 ± .08	.92 ± .07
ATP	2.58 ± .07	2.62 ± .07	2.84 ± .11
Total nucleotides	4.06 ± .14	4.05 ± .11	4.20 ± .15

*Difference from control significant at P = .01 level.

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Some aspects of oxidative metabolism of glucose in brain suspensions

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PRELIMINARY STUDIES of the interrelations between carbohydrate and amino acid metabolism in brain have confirmed and extended previous observations reporting the presence of oxidative metabolism of glucose by brain suspensions. The tissue preparations from brain and liver were incubated in a saline medium, either with glucose-6-phosphate or ribose-5-phosphate. It was found that 90 minutes after incubation a considerable amount of the disappearing substrate could be accounted for by the appearance of a pentulose and a heptulose. The phosphate esters of these two carbohydrates were isolated from the incubation mixture, as described by Horecker, Smyrniotis, and Klenow.[1] The elution of the phosphate from the Dowex I column indicated the presence of ribulose-5-phosphate in the incubation mixture. The heptulose was identified as sedoheptulose phosphate, using the specific orcinol test described by Dische.[2]

The optimum absorption peak was between λ 605-610, which agrees with the one given by an authentic sample of sedoheptulosan monohydrate (kindly supplied by Dr. B. L. Horecker and Dr. N. K. Richtmeyer). The final confirmation of the formation of sedoheptulose phosphate from glucose or ribose phosphate was obtained on paper chromatograms developed in 80 per cent phenol, acetone-water (10:3), and acetic acid-butanol-water (1:4:5). For this purpose the reaction mixture after 90 minutes incubation was dephosphorylated at pH 5.0 with potato phosphatase, prepared according to an unpublished method of Kornberg. The Rf values of the sugars in the acetic acid-butanol-water system were as follows:

	Authentic Sample	*Incubation Brain*	*Mixture Liver*
Glucose	0.21	0.21	0.20
Sedoheptulose	0.26	0.26	0.25
Ribose	0.33	0.33	0.32

The sugars were detected as white spots against blue background on paper chromatograms after a spray with 0.1 per cent periodic acid, followed by the benzidine reagent. The control system, which contained $MgCl_2$ and cysteine, gave a rapid conversion of ribose-5-phosphate into sedoheptulose phosphate. The addition of various co-factors, such as adenosine triphosphate (ATP), thiamine pyrophosphate (TPP), and triphosphopyridine nucleotide (TPN), showed no considerable effect on the formation of sedoheptulose. The experiments also suggested that the transketolase system of the tissue preparations, which is responsible for the conversion of the pentose into the heptose, was not, to any appreciable extent, dependent on the exogenous supply of thiamine pyrophosphate. In a typical experiment, 200 μM of ribose-5-phosphate were incubated with brain and liver homogenate in a control glycylglycine buffer medium (pH 7.5) fortified with ATP, TPN, and TPP. At the end of 90 minutes the incubation mixture contained 14.5 μM of sedoheptulose per mg. nitrogen of brain homogenate, and 12.8 μM of sedoheptulose per mg. nitrogen of liver homogenate.

The demonstration of the conversion of glucose or ribose into a heptulose has added interest to the metabolic studies of this com-

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pound and of shikimic acid. The possible relationships of 2-keto-3-deoxy-7-phospho-D-glucoheptonic acid[3] to 5-dehydroshikimic acid and of shikimic acid to the biosynthesis of phenylalanine, tryptophan and p-aminobenzoic acid[4] have prompted us to undertake preliminary investigation on shikimic acid as well. It was found that TPN-dependent oxidation of glucose by brain homogenate was considerably reduced (86 per cent) in the presence of shikimic acid, but not in the presence of quinic acid. Further studies are in progress to evaluate the significance of this observation in the biosynthesis of amino acids in the brain.

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Metabolic studies of gamma-aminobutyric acid

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Since the first report of the unique presence of large quantities of γ-aminobutyric acid (GABA) in mouse brain,[1] a considerable body of biochemical and physiologic data has indicated that this substance may play an important role in cerebral metabolism.[2,3] GABA has been found in an easily extractable form in large amounts in the central nervous system of representative members of fish, amphibian, reptilian, avian, marsupial, and mammalian species. It was the only easily extractable ninhydrin-reactive constituent found on two-dimensional paper chromatograms which showed progressive increases at successive developmental stages in extracts of brains of chick embryo, mouse, salamander, and frog. In the mouse the period of greatest increment in features related to maturation of the nuclear masses and fiber tracts of the central nervous system corresponds to the attainment of the highest concentration of GABA and the maximal activity of glutamic acid decarboxylase (GAD), the enzyme which forms GABA from glutamic acid. In an extensive survey of mammalian tissues, GABA and GAD have been detected only in brain and spinal cord, and higher levels have been found in gray matter than in white matter. GABA was found to be taken up readily by liver, kidney, and muscle in the mouse after parenteral administration, but significant increases were not noted in the content of GABA in brain.

The known metabolic relationships of GABA in brain are shown in schematic form in figure 1. It is apparent that intimate relationships exist between cellular oxidations and the metabolism of GABA and glutamic acid. GABA is formed in brain from glutamic acid by the action of GAD. GABA transaminase (GABA-T), an enzyme found in brain as well as in other tissues and in microorganisms, catalyzes the transamination of GABA with α-ketoglutarate (*a* KG). Since *a* KG, glutamic

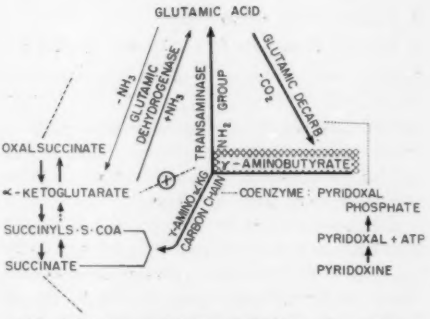

Fig. 1. Known metabolic relationships of γ-aminobutyric acid in brain.

acid, GAD, GABA, and GABA-T are found in the central nervous system, there exists the possibility of a shunt around the *a* KG oxidase system by which *a* KG could be withdrawn as glutamic acid by the donation of the amino group of GABA, and the carbon chain of GABA could enter the tricarboxylic acid cycle at the succinate level. Although the enzymatic details are still being worked out, experiments in which GABA-1-C^{14} was administered intraperitoneally to rats or intracerebrally to mice or incubated with minces of mouse brain gave results consistent with the above formulation.

It has been shown that GAD requires pyridoxal phosphate (PP) as a coenzyme, which can be synthesized in brain from adenosine triphosphate and pyridoxal. A suitable assay procedure for the measurement of GABA-T activity has now been developed. It depends on the differential chelation of copper by small amounts of glutamic acid in the presence of large amounts of GABA and *a* KG in alkaline solution. Data showing activation

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TABLE 1

STIMULATION OF GABA TRANSAMINASE OF BEEF BRAIN BY PYRIDOXAL PHOSPHATE

Type of Preparation	Glutamic Acid Formed μM/mg. Protein/Hr. $-B_6$	$+B_6$	Stimulation by B_6 Per Cent
1. Aqueous extracts of brain acetone powders			
A) 1 acetone washing	0.48	0.56	17
B) 2 acetone washings	0.52	0.68	31
C) acetone-butanol-acetone washing	0.40	0.56	40
2. Extract frozen for 48 hours	0.14	0.28	100
3. Solution of dialyzed 66% $(NH_4)_2SO_4$ precipitate	0.10	0.23	130

Each tube contained 0.2 ml. of enzyme preparation; 1.0 ml. of borate buffer, pH 8.1; 0.1 ml. containing 40 μM αKG; 0.1 ml. containing 40 μM GABA; and pyridoxal phosphate, 20-γ, when added. All values suitably corrected for blanks.

of the GABA-T by PP are shown in table 1. Work to date indicates that the apoenzyme of GAD has a lower degree of affinity for the coenzyme than that of GABA-T.

Extensive studies by Florey[4] on a factor in beef brain which has potent physiologic activity in a number of test systems have culminated in the identification of this factor as GABA,[5] and considerable evidence has been adduced in favor of assigning to it the role of a transmitter substance of inhibitory neurons. Convulsive seizures are known to occur in experimental animals and humans suffering from pyridoxine deficiency. Dietary pyridoxine deficiency of insufficient severity to produce seizures in rats resulted in a marked but readily reversible decrease in the activity of GAD, the enzyme producing GABA.[2,3] Killam[6] has shown that convulsant hydrazides, compounds which react with PP, produce decreases in the activity of GAD and the content of GABA in the brains of rats, and a good correlation was obtained between the electrical evidence of seizure activity produced by these substances and the actual decrease in GAD activity in the affected areas in brains of cats. Isolated hydrazide-treated plugs showed seizure activity only when stimulated. Seizures induced by administration of hydrazides to cats have been reversed by GABA.[7] The above data fit the hypothesis that seizure activity in pyridoxine deficiency and in hydrazide poisoning results from a decreased activity of GAD, with a consequent decrease in the ability to produce GABA, a substance of importance in the action of inhibitory neurons in the central nervous system. The consequent failure to regulate or modify properly the activity of the excitatory neurons would tend to produce an indiscriminate spread of electrical impulses with resultant seizures. It appears possible that the GAD–GABA–GABA-T system might be a companion system to the choline acetylase–acetylcholine–cholinesterase system in the central nervous system (see reference 8 for discussion of role of acetylcholine in seizures). The balance between these systems, rather than the absolute amount of any single substance or enzymatic activity, might be important in the regulation of activity in a given region of the central nervous system. We may begin to analyze the action of a number of pharmacologically active materials in terms of the imbalances which would be created by their action on specific enzymes in the γ-aminobutyrate and acetylcholine sequences.

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Studies with tritium-labeled inositol

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Inositol is of interest to the neurochemist principally for two reasons: first, the presence in the brain of a unique inositol compound, diphosphoinositide,[1] and second, the more recent finding of Dawson[2] that the inositol-containing fraction of the phospholipids of many tissues exchanges its phosphorus with inorganic phosphate at a very high rate. Hokin[3] has shown that this exchange is stimulated by acetylcholine and also that free inositol exchanges with phospholipid inositol in tissue slices.

Since no adequate technics for chemical determination of inositol were available, a method was developed involving spectrophotometric measure of periodate consumption.[4] Using this method, it was seen that, with a single exception, incubated guinea pig tissue slices of various organs released free inositol into the incubation medium and that the addition of free inositol prevented this release. Only in kidney slices was a net disappearance of inositol observed.

Inositol labeled with tritium in the 2 position (inositol-2-T) was prepared by a method analogous to the preparation of inositol-2-D.[5] Following intraperitoneal injection of inositol-2-T, a rat was sacrificed and trichloroacetic acid precipitates of the organs were made which were then extracted with a chloroform-methanol mixture. This lipid extract was found to contain radioactivity in the following order of decreasing activity/gram wet weight of tissue: kidney > pancreas > heart > liver > spleen > lung > testis > diaphragm > brain. Hydrolysis of such an extract revealed that the radioactivity was present as inositol.

The chloroform-methanol insoluble residue was also studied and a considerably different sequence of incorporation of inositol was noted: testis > spleen > kidney > pancreas > heart > diaphragm > lung > liver > brain. The nature of the radioactive product in the methanol-chloroform-insoluble residue has not as yet been identified.

An anionic material which was soluble in trichloracetic acid was also detected. The highest concentration was found to be in kidney. Subsequent studies revealed that the clear supernatant solution from a broken-cell preparation of kidney was able to convert inositol to the anionic material. The product was purified by gradient elution chromatography from Dowex-1 formate and found to possess no inositol or phosphate. A positive carbazole test was noted, indicating that this material might be identical with the DL-glucuronic acid recently isolated by Charalampous.[6]

Tritium-labeled water (THO) production was studied in tissue preparations. Following incubation, water was recovered by distillation and counted by liquid scintillation technics. Brain, testis, and kidney slices produced THO from inositol-2-T, but in cell-free preparations THO was recovered only from kidney.

Incorporation of inositol into lipid was studied in kidney preparations. It was found that mitochondria were capable of incorporating inositol into the chloroform-methanol extract of a trichloroacetic acid precipitate when fortified by heat-stable factors from the supernatant solution.

Recent studies reveal that the supernatant factors can be replaced by ATP, Mg^{++}, *a*-ketoglutarate, and a cytidine nucleotide.

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Studies on the biosynthesis of cerebroside galactose

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IT IS WELL ESTABLISHED that cerebrosides are an integral component of myelin in mammals, and data on the synthesis of cerebrosides may, therefore, contribute to the broader problem of myelin deposition. The studies to be presented are concerned particularly with cerebroside synthesis as a function of age. In addition, certain observations will be presented on the metabolic origin of cerebroside galactose carbon from glucose carbon. In the process of making these observations, it was also possible to obtain data on the incorporation of glucose carbon into several brain lipid fractions.

Folch and his associates[1] have published detailed data on the composition of the brain in relation to maturation in Swiss Albino mice. It was decided to use mice of a similar strain in all our experiments, and hence the rate of lipid synthesis, as measured by the incorporation of radioactive glucose carbon, could be compared with the known changes in concentration of these lipid components during maturation.

Glucose-C^{14}, either uniformly labeled or labeled exclusively in the 6 position, was used as the radioactive precursor. A small measured volume of a solution containing the radioactive glucose in physiologic saline was injected intraperitoneally into the experimental animals. Each animal received 0.1 mg. of glucose per gm. of body weight. This quantity contained about 0.2 microcurie of C^{14}. After a suitable time interval the animals were killed by decapitation. The brain was removed rapidly and homogenized immediately with 20 volumes of 2:1 chloroform-methanol (v/v).[2] The filtered extract was washed once with water and four times with calcium chloride-containing "theoretical upper phase," as described by Folch.[3] A flow sheet of our fractionation procedure is shown in figure 1. The methanol-water upper phases obtained were taken to dryness by vacuum distillation, dialyzed for five days against water, and the contents of the dialysis bag were lyophilized. The specific activity of the strandin thus obtained was then measured.

The lipids in the chloroform phase were subjected to acid hydrolysis, using Svennerholm's method.[4] The material was then filtered; hexose analysis by Svennerholm's orcinol method[4] was carried out on an aliquot of the filtrate. A known amount of carrier galactose was then added, the 2,5-dichlorophenylhydrazone prepared, and its specific activity measured.

The acid insoluble residue remaining on the filter following hydrolysis was dissolved in chloroform-methanol and again purified using Folch's method,[3] the aqueous phase being discarded. The chloroform phase was taken to dryness, dissolved in ether, and partitioned between ethyl ether and 0.5 N sodium hydroxide. From the ether phase, cholesterol was precipitated as its digitonide and counted. The aqueous phase was acidified, and the fatty acids were extracted with ether and counted.

In order to select the most favorable time interval between injection and sacrifice, radioglucose was administered to 22 day old mice, and the animals were killed one-half to seven hours after injection. The specific activity of the brain total lipids was determined at each time interval, while the cerebroside galactose specific activity was measured on pooled samples representing one-half and one hour, two and three hours, and four and seven hours after injection of the isotope. It was noted that the peak specific activity for total lipid was reached in two hours, while the specific

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**Work performed during the tenure of a Lederle Medical Faculty award.

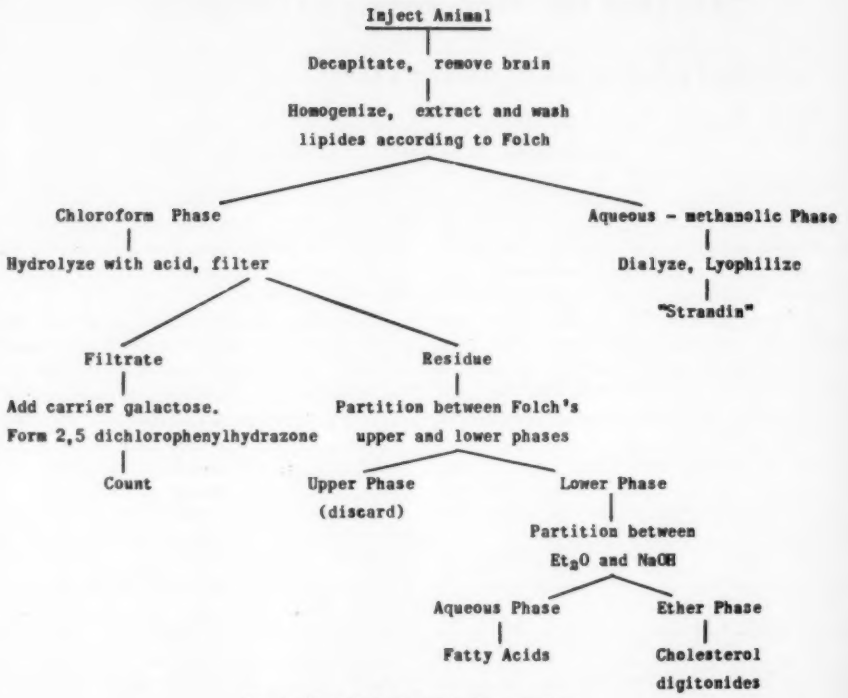

FIG. 1. Flow sheet of fractionation procedure.

activity of cerebroside galactose appeared relatively constant. For reasons which are unknown at present, the time curve presented here shows a considerably earlier peak than that obtained in rats by Radin and collaborators[5] and by Burton and collaborators.[6]

In measuring the effect of age on brain lipid synthesis, glucose-U-C^{14} was used as the radioactive precursor and the time between injection and sacrifice was one hour. The specific activities of brain total lipid and fatty acid in mice from three days to 180 days old were compared. It was found that the shape of the two curves was similar, and that there appeared to be a peak in specific activity in animals eight days old. The activity diminished thereafter. It was noted that between eight days and 22 days the curves were somewhat irregular. It is believed that this is due at least in part to difficulty in estimating dosage of radioglucose with any precision. The dose had been selected on the basis of a constant amount of glucose per gram of body weight for all age groups. However, during the period under study, body weight and composition are undergoing marked changes. Thus, in our mice, body weight more than doubled between 16 and 22 days of age. It would be ideal to select dosage conditions in such a way that one would achieve the same blood glucose specific activity in each animal, and it is quite possible that the method employed here did not achieve this. In order to plot total lipid, cerebroside galactose, and cholesterol specific activities on the same graph, a semilog scale had to be employed. It was noted that cerebroside galactose specific activity was higher than that of any other fraction studied and reached a peak at eight days. It then fell precipitously to much lower levels. While the specific activity of total lipid at eight days was about six times higher than at 180 days, the corresponding drop for cholesterol was 19-fold and for cerebroside galac-

tose it was 160-fold. The total strandin specific activity was determined. It was of the same order of magnitude as that of total lipids, and it continued to maintain a significant level even in the 180 day old animals. The ratio between the eight day and 180 day specific activity was 5.4. The results presented so far represent specific activity data. In order to reach conclusions as to the rate of synthesis of various components, the concentration of these lipids has to be taken into consideration. During the age period covered by our experiments, the brain cerebroside galactose concentration on a wet weight basis increased 100-fold. By multiplying cerebroside galactose specific activity by its concentration, an index of the rate of synthesis can be obtained, and this calculation reveals that animals 16 days old exhibit a sharp maximum ability to incorporate glucose carbon into galactose. Folch has found, from the change in concentration of cerebroside galactose, that maximum synthesis occurs between 19 and 22 days.[1] The isotope methods discussed here will be extended to groups of animals between 16 and 22 days in order to focus more precisely on the age at which cerebroside synthesis is maximal.

Finally, an experiment was performed in which it was possible to obtain sufficient incorporation of glucose into cerebroside galactose to make degradation of the galactose possible. In this experiment, glucose-6-C^{14} was used as the radioactive precursor. Following hydrolysis of the cerebrosides, an amount of nonradioactive glucose was added equal to the total hexose content of the hydrolysate. Glucose and galactose were then separated by paper chromatography, using water-pyridine-n-butanol (3:4:6) as the solvent. The glucose isolated contained, at most, 17 per cent of the radioactivity recovered; the galactose portion contained the remaining 83 per cent.

Both an aliquot of cerebroside hexose prior to chromatography and the purified galactose were degraded. It was noted that there was little, if any, randomization of the label, that is, the cerebroside galactose contained almost all (95 per cent) of the activity in carbon 6. This finding suggests that glucose enters cerebroside galactose as a unit, and is consistent with the mechanism for interconversion of glucose and galactose proposed by Leloir[7] and Anderson[8] and with the results of Burton and associates.[6]

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Studies on the biosynthesis of galactolipids

Robert Main Burton, Ph.D., Mary Ann Sodd, M.S. and Roscoe O. Brady, M.D.

MELTZER[1] and Weiss[2] have observed that the perfusion of brain with acetate-1-C^{14} or octanoate-1-C^{14} results in the incorporation of C^{14} into cerebral lipids. Radin and co-workers[3] and Moser and Karnovsky[4] have found that the parenteral administration of C^{14}-labeled hexoses to the rat or mouse results in labeling of the glucolipids of brain. Studies in our laboratory have shown that both D-glucose-U-C^{14} and D-galactose-1-C^{14} can be readily incorporated into the neutral galactolipid fraction of rat brain tissue, *in vivo*.[5] The neutral galactolipid fraction was isolated by a modification of the procedure developed by Radin and co-workers.[6] This lipid fraction probably consists of cerebrosides, but absolute identification has not been made as yet. The rate of incorporation of the hexoses was correlated with the age of the animal, and maximum specific activity of the galactolipid fraction was found in rats which were from 10 to 20 days old. Following a single injection of the C^{14}-carbohydrate, the specific activity of the cerebroside fraction increased until a maximum was reached in about eight hours. Thereafter, the radioactivity of the neutral galactolipids decreased during a 24-hour period to approximately 30 per cent of the maximum specific activity observed.

Preparations of rat brain tissue have been shown to contain enzymes which are capable of catalyzing the *in vitro* incorporation of the sugar moiety into the neutral galactolipid fraction. These enzymes appear to be most active in young rats about two weeks of age. It has been found that, upon fractionation of the cell-free preparations of brain tissue according to the procedure of Brody and Bain,[7] the microsomal fraction (R_4) contains most of the enzymatic activity necessary for incorporating galactose or glucose into the galactolipid fraction. The incorporation of the free hexose was observed to be dependent upon the addition of adenosine triphosphate (ATP). However, in the absence of ATP, both 1-C^{14}-D-galactose-1-phosphate (galactose-1-PO_4=) and uridine diphospho-D-galactose-1-C^{14} (UDPgalactose) could be incorporated into the galactolipid fraction. Recent experiments suggested that the incorporation of galactose plus ATP and of galactose-1-phosphate required the presence of uridine diphosphoglucose (UDPglucose). These various observations are consistent with the sequences of events illustrated by equations 1 through 7.

Studies demonstrating the presence in brain tissue of the various enzymes associated with the activation of glucose and galactose (equations 1, 2, and 6) have recently been reviewed by a number of authors, such as McIlwain[8] and Racker.[9] The presence of UDPglucose pyrophosphorylase activity (equation 3) and UDPgalactose-4-epimerase activity (equation 4) in preparations of brain tissue has been shown.[10] Kurahashi[11] has demonstrated galactose-1-phosphate uridyl transferase activity in brain preparations (equation 7).

Degradation of the neutral galactolipids obtained from these experiments has revealed that the incorporated radioactivity resides in the sugar moiety. The identity of the lipid acceptor is not yet known but is under study.

Preliminary examinations of the sugar obtained from the radioactive glycolipid indicate that it is mainly galactose. It is of considerable interest to establish the identity of the sugar moiety, since a preponderance of glucocerebroside rather than galacto-cerebroside is found in conditions such as Gaucher's disease.

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I. The incorporation of glucose:

A. Glucose is activated by ATP in the presences of hexokinase and phosphoglucomutase (equations 1 and 2)

$$\text{Glucose} + \text{ATP} \xrightarrow[\text{hexokinase}]{} \text{glucose-6-PO}_4^{=} + \text{ADP} \quad (1)$$

$$\text{Glucose-6-PO}_4^{=} \underset{\text{phosphoglucomutase}}{\rightleftharpoons} \text{glucose-1-PO}_4^{=} \quad (2)$$

B. The glucose-1-phosphate is incorporated into a uridylic nucleotide and converted to UDPgalactose (equations 3 and 4).

$$\text{Glucose-1-PO}_4^{=} + \text{UTP} \underset{\text{UDPglucose pyrophosphorylase}}{\rightleftharpoons} \text{UDPglucose} + \text{pyrophosphate} \quad (3)$$

$$\text{UDPglucose} \underset{\text{UDPgalactose 4-epimerase}}{\rightleftharpoons} \text{UDPgalactose} \quad (4)$$

C. The UDPgalactose may donate the galactose moiety to a lipid acceptor to form the neutral galactolipid (equation 5).

$$\text{UDPgalactose} + \text{lipid acceptor} \longrightarrow \text{galactolipid} + \text{UDP} \quad (5)$$

II. The incorporation of galactose:

A. Galactose is activated by ATP and incorporated into the uridylic nucleotide (equations 6 and 7).

$$\text{Galactose} + \text{ATP} \xrightarrow[\text{galactokinase}]{} \text{galactose-1-PO}_4^{=} + \text{ADP} \quad (6)$$

$$\text{Galactose-1-PO}_4^{=} + \text{UDPglucose} \underset{\text{galactose-1-phosphate uridyl transferase}}{\rightleftharpoons} \text{UDPgalactose} + \text{glucose-1-PO}_4^{=} \quad (7)$$

B. The UDPgalactose formed can then donate the galactose moiety to form the galactolipid (equation 5).

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Biochemical comparison of Wallerian degeneration in the central peripheral nervous systems

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WALLERIAN DEGENERATION in the distal segment of cut peripheral nerves has long been a subject of great interest to histologists. In more recent years this degenerative process has been studied using chemical methods. The biochemical characterization of this process, culminating in the comprehensive quantitative studies of Rossiter and his co-workers,[1,2] has greatly increased our knowledge of the process of demyelination in the peripheral nervous system. Because of the known differences in degeneration and regeneration of the peripheral and central nervous systems, it seemed of importance to investigate the biochemical nature of this process in the central nervous system and to compare it with that in the peripheral nervous system.

In this study the optic and tibial nerves of the rabbit were examined chemically after 15, 45, 100, and 200 days of degeneration. Correlated electron microscopy, histology, and enzymatic studies were also carried out on the same nerves and will be reported later.

The methods of preparing and analyzing the tissue are those of Lowry and co-workers[3,4] and of Robins and co-workers.[5] These micromethods allow the quantitative estimation of various phosphorus fractions, lipid components, and desoxyribonucleic acid on a single sample of nerve weighing 10 to 40 micrograms dry weight.

In recording results the values are based on fat-free dry weight so that the decreasing amount of lipid, which composes some 50 per cent of dry weight in the normal nerves, will not distort the true picture of the degenerative process. The results so obtained on the degenerating nerves are presented in table 1. The various lipid components of the optic and tibial nerves are qualitatively and quantitatively similar, except for the lower value for sphingomyelin in the optic nerve. In the tibial nerve all of the lipids, with the exception of total cholesterol, have dropped to 50 per cent or less of their normal value by 15 days, and by 100 days only about 10 per cent of the lipid still remains. The quantitative and temporal nature of these results are in essential agreement with those reported by Johnson and co-workers[1] in the sciatic nerve of the cat. The changes in total acid soluble phosphorus and the desoxyribonucleic acid are similar to previously reported findings.[2] The temporal changes in the lipids of the degenerating optic nerve present a striking contrast to those of tibial nerve. The lipids of the degenerating optic nerve show no significant decrease at 15 days and even at 100 days have only decreased to 50 per cent of their initial value. At 200 days 30 per cent of the initial amount of lipid still remains; this corresponds to the stage found in the tibial nerve between 14 and 45 days. Thus it appears that the depletion of lipids in the optic nerve is about five times slower than that of the tibial nerve.

The slow steady increase in the desoxyribonucleic acid of the optic nerve presents quite a different picture from that found in the tibial nerve. These differences in the changes in desoxyribonucleic acid of the two nerves are to be expected from the differences in the cellular changes observed in histologic studies.

In conclusion, Wallerian degeneration in the central nervous system is remarkably different from degeneration in the peripheral nervous system. The biochemical and histologic basis for these differences will be the subject of further study.

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TABLE 1

CHANGES IN CHEMICAL CONSTITUENTS DURING DEGENERATION

Nerve	Constituent	Control Value	Days of Degeneration 14	45	100	200
		(mM./kg. of fat-free dry weight)		(Per Cent of Control)		
Tibial	Cephalins	768	37	13	5	
	Lecithins	200	47	39	17	
	Sphingomyelin	458	41	20	5	
	NPSL*	361	50	25	3	
	Total cholesterol	1190	72	36	10	
	Acid soluble P	132	139	114	101	
	DNA	5†	380	233	207	
Optic	Cephalins	550	89	81	44	33
	Lecithins	256	88	108	66	46
	Sphingomyelin	177	107	113	51	36
	NPSL*	300	99	103	63	17
	Total cholesterol	985	94	122	75	32
	Acid soluble P	200	109	124	129	112
	DNA	9†	134	161	182	245

*Nonphosphorus-containing sphingolipids, chiefly cerebrosides.
†Gm. per kg. fat-free dry weight.

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Studies on myelination and demyelination: Lipogenesis by nervous tissue *in vitro*

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LIPID BIOSYNTHESIS by nervous tissue *in vitro* was studied by incubating samples of gray matter, white matter, and peripheral nerve of rats and hens in Warburg flasks, in Krebs-Ringer-phosphate medium with glucose (10 μM/ml.), in the presence of labeled building blocks of lipids (acetate-1-C^{14}, choline-1,2-C^{14}, glycerol-1-C^{14}, glucose-U-C^{14}, and phosphate-P^{32}). After incubation for four hours the tissues were homogenized in 2:1 chloroform-methanol, the total lipids were extracted and washed by the method of Folch,[1,2] and were counted in a windowless gas-flow counter. The preparations of peripheral nerve from the rat consisted of the intact sciatic and brachial plexus; and from the hen the sciatic deprived of the perineurium. White matter was prepared from the spinal cord, either by stripping off funiculi or by halving the cord and carefully wiping out the gray matter. Gray matter was shaved off the surface of rat cerebrum with the aid of a hand-microtome[3] set to cut at 0.22 to 0.23 mm. All preparations were examined histologically.

STUDIES ON NORMAL TISSUES

The various preparations were compared with respect to several constants, including oxygen uptake and incorporation into the lipids of the label of the different substrates. In gray matter and peripheral nerves, both respiration and lipogenesis continued at almost linear rates throughout the incubation period; white matter was not as durable a preparation. Mechanical trauma, delay in processing, and cholinesterase inhibitors added *in vitro* invariably depressed lipogenesis from acetate to a greater extent than oxygen uptake.

Changes related to age were studied in detail. In the peripheral nerves of newborn rats, oxygen uptake is almost as high as in cerebral cortex at the same age (71 μl. O_2 per 100 mg. fresh weight per hour). It rises for about ten days, then drops progressively for 150 days, with only slight change thereafter. Lipogenesis from acetate also follows a biphasic curve: it rises to 162 per cent of the value for newborn animals between the first and fifth day, returns to the initial value (100 per cent) by 12 days, then drops to one twenty-fifth (4 per cent) of this value in the course of 150 days. The initial rise corresponds to the period during which myelin first becomes demonstrable histologically in the sciatic nerves.

In the cerebral cortex of the rat, oxygen uptake rises sharply after birth and reaches a constant rate (225 per cent of the value for the newborn animal) at 40 days. During the same period, lipogenesis from acetate drops to 6 per cent of the level for the newborn. If respiration and lipogenesis are plotted as a function of age, the curves pertaining to brain show an initial steep slope, followed by a sharp bend at 40 days; those related to nerve are more protracted and tend to plateau at five months. The same general patterns were obtained when the weight of the brain and of the peripheral nerves, respectively, was plotted as a function of age. Thus, the rates of the metabolic functions investigated follow closely the pattern of growth rate characteristic of each given organ.

In the peripheral nerve of the normal adult

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*Work performed during the tenure of a Lederle Medical Faculty award.

rat several proximo-distal gradients were observed. If the upper half of the sciatic nerve is compared with the tibial nerve, the latter is found to contain 20 per cent less lipid, its oxygen uptake is 13 per cent lower, and acetate incorporation into the lipids is also 15 per cent lower. These findings may be of relevance in the pathogenesis of the "peripheral neuropathies."

Wallerian degeneration

Bilateral section of the sciatics was performed in 128 rats. The animals were killed at intervals varying between four hours and 50 days; particular attention was given to the early stages of degeneration. Oxygen uptake (referred to dry weight) was reduced to 75 per cent of normal after 24 hours, then increased to 200 per cent by the tenth day. Incorporation of acetate into the lipids dropped to 30 per cent of normal during the first five days; by the end of four weeks it rose above 300 per cent. This result in rats with acetate-C^{14} is similar to that observed by Magee and Rossiter[4] using radioactive phosphate. In the proximal stump, excluding the regenerating cone, incorporation of acetate was also reduced by approximately one-third 12 days after section.

Demyelination induced by Mipafox

Rats treated with biweekly subcutaneous injections of Mipafox (bis-monoisopropylaminofluorophosphine oxide) over three to four months developed weakness of the hind limbs and a typical peripheral neuropathy characterized by degeneration, histologically of Wallerian type, in the distal half of the tibial nerve. At this stage, lipogenesis from acetate was found to be depressed in the upper half of the sciatic and enhanced in the tibial nerve (similar to the picture in early and late stages of Wallerian degeneration, respectively). In hens killed 20 to 25 days after injection of the same drug, demyelination of white matter was studied on isolated funiculi stripped from the spinal cord. Acetate incorporation into the lipids was depressed in direct proportion to the extent of demyelination observed histologically within the tracts. In the sciatic nerves of the same hens acetate incorporation was reduced to 43.7 per cent ($P < 0.01$) of normal.

These studies on the patterns of lipogenesis in nervous tissue, using various lipid building blocks, are continuing. Subjects currently being investigated are: the changes in longitudinal tracts of the spinal cord of the rat at varying intervals after surgical section, and the interference with lipogenesis by various chemical agents *in vitro*.

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Mode of action of an inhibitor of acetylcholine synthesis

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THE HEMICHOLINIUMS[1] are quaternary bases characterized chemically by the incorporation of a choline (or choline-like) moiety into a six-membered ring through hemi-acetal formation. They are highly toxic, producing respiratory paralysis of central origin, which is delayed in onset and can be prevented by the administration of either eserine or choline. On the basis of these and other findings, Schueler[1] suggested that the hemicholiniums act by interfering with some cholinergic mechanism. Experiments on perfused ganglia and minced brain have shown[2] that one of these compounds, HC-3 (No. 3 of Schueler's list[1]), strongly inhibits the synthesis of acetylcholine by nervous tissue, except when excess of choline is present in the extracellular fluid. In the ganglion preparation the concentration of HC-3 necessary to produce a well-marked inhibition is of the same order as the concentrations found in the plasma of poisoned animals. The following observations on intact animals show that this drug produces pharmacologic effects that may well be ascribed to interference with acetylcholine synthesis.

Anesthetized cats maintained on artificial respiration can be given doses of HC-3 that would otherwise be lethal without obvious circulatory impairment. In such animals transmission over autonomic pathways that include one or more cholinergic junctions is blocked by HC-3, but only when the pathways are made to conduct impulses at high frequency for several minutes. The block is delayed in onset, but progresses to completion; it can be relieved by slowing the frequency of stimulation, or more rapidly by the injection of choline in doses which would have no significant effect on unpoisoned animals. Observations of this sort have been made on the cardiac vagal efferents, the chorda tympani supply to the salivary gland, and the sympathetic ganglion. In contrast, the response to prolonged high-frequency stimulation of adrenergic fibers is unaffected by HC-3. These results are compatible with the idea that HC-3 has no effect on the mechanisms involved in the release of acetylcholine, but blocks transmission in active cholinergic pathways by allowing the presynaptic stores of transmitter to be depleted. It is attractive to suppose that the toxic action of the drug is also due to an effect of this sort, even though it has not been shown that the transmission of respiratory activity within the central nervous system requires the participation of cholinergic neurons.

While these findings suggest that the characteristic pharmacologic activity of HC-3 depends on its ability to inhibit acetylcholine synthesis, they do not elucidate the nature of its inhibitory action. The drug appears to be only a weak inhibitor of choline acetylase (that is, the enzyme that transfers acetyl groups from coenzyme A to choline), as shown by its failure to prevent the synthesis of acetylcholine by extracts of acetone powders of brain incubated with choline and acetyl-coenzyme A.[2] That it also fails to inhibit the acetylation of coenzyme A is indicated by the finding that pigeon liver homogenates can acetylate sulfanilamide normally in the presence of high concentrations (up to 10^{-2} M) of HC-3.[2] There is, however, another possible way in which HC-3 could inhibit acetylcholine synthesis by nervous tissue, namely by interfering with the supply of choline to the acetylating enzyme.

The ultimate source of choline for the manufacture of acetylcholine must be the blood, but this source may be barely sufficient when acetylcholine turnover at nerve endings is

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maintained at a high rate for a long time. For example, we have observed that in a repetitively stimulated superior cervical ganglion with its circulation intact (the animal being eserinized), the output of acetylcholine into the venous blood remained steady at about 10^{-10} M/min., while the arterial blood supplied no more than 4×10^{-10} M/min. of free choline. These figures suggest that choline can be transferred with remarkable efficiency from the extracellular fluid to the nerve endings, which is the more surprising since choline ordinarily does not penetrate very readily into neurons.[3] Can it be that a special choline carrier in the presynaptic membrane brings choline to the intraneuronal sites of acetylation?

For technical reasons this question cannot easily be answered by experiments on the ganglion preparation, but relevant evidence has been supplied by experiments on the avian kidney. This organ is known to excrete choline (and certain other bases) by a tubular mechanism different from the one involved in the excretion of p-aminohippuric acid, phenol red, and the like.[4] If HC-3 prevents acetylcholine synthesis by interfering with choline transport, it might be expected also to inhibit choline excretion by the avian kidney. This expectation has been confirmed in the following way. A solution containing choline (5×10^{-4} *M*) and phenol red (3×10^{-4} *M*) was infused into one renal portal vein of an anesthetized chicken at the rate of 1.0 ml./min. while urine was being collected from each ureter. The urinary output of each substance remained high and steady, choline being excreted mainly by the ipsilateral kidney. When HC-3 (2×10^{-4} *M*) was added to the infusion fluid, the output of choline was sharply diminished, while the output of phenol red remained unchanged. When infusion of the original mixture was resumed the output of choline rose again. Figure 1 shows the results of a typical experiment.

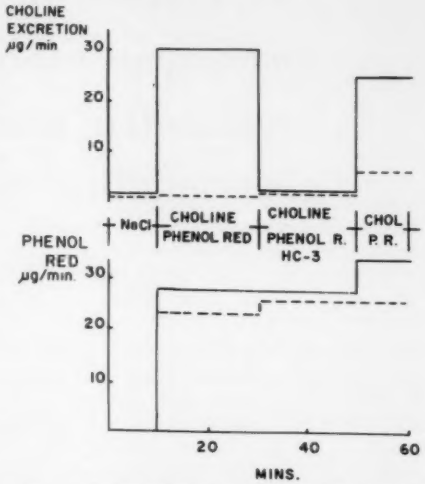

FIG. 1. Selective inhibition by HC-3 of choline excretion by the avian kidney. Rooster, 2.1 kg.; continuous infusion of choline and phenol red with or without HC-3 into renal portal vein during periods indicated. Continuous lines: output of choline (as chloride) and phenol red from ipsilateral kidney. Broken lines: output of same substances from contralateral kidney.

These findings demonstrate that HC-3 strongly inhibits the transfer of choline across the cells of the avian renal tubule, a process which, as far as is known, does not involve the acetylation of choline. They make it highly probable that HC-3 interferes with acetylcholine synthesis in nervous tissue by competitively inhibiting the transport of choline to its intracellular site of acetylation.

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Correlation of rate of compulsive circling in rabbits with the asymmetric accumulation of acetylcholine in cerebral cortices

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THE PHENOMENON of compulsive circling is the involuntary or forced turning of an animal in circles. The type of coordinated movement may vary from pivoting on the hind legs to moving along the circumference of a small circle. Such compulsive behavior may be produced experimentally in a number of ways. One such method is by injecting the anticholinesterase drug, diisopropyl fluorophosphate (DFP), into one common carotid artery. When this is done in the rabbit, one of three different behavioral states may be noted.[1–3] The majority of the animals circle away from the injected side ("lefters"), fewer are unaffected by the drug and maintain their normal mode of progression ("neutrals"), while some circle toward the injected side ("righters"). In addition to the following changes, such as constriction of the right pupil, chewing movements, tremors, head and eye nystagmus, it was also noted that the number of turns made by the rabbits in a unit of time varied considerably.

After a study was completed in which the cholinesterase (ChE) activity was determined in the left and right areas of the frontal cortex, caudate nucleus, thalamus, superior and inferior colliculus, medulla oblongata, pons, and cerebellum (flocculonodular and lingular lobes), it was concluded that the frontal cortex and caudate nucleus were the only two areas of the brain which appeared to be directly involved in the production of the circus movements produced by the unilateral intracarotid injection of DFP.[3] The enzyme study was followed by one in which the free or soluble acetylcholine (ACh) was measured in the left and right motor cortex and caudate nucleus.[4,5] In rabbits circling to the left, the ACh content was higher on the right side in both the cortex and caudate nucleus. However, in animals turning compulsively to the right, the left cortex had a higher ACh concentration than the opposite side. In the case of the caudate nuclei, the right side was higher than the left. Since the ChE data as well as the ACh data for the caudate nuclei in "righters" and "lefters" show little difference, it is difficult to implicate the caudate alone in the mechanism of compulsive turning. It is thought that the caudate nucleus may be involved in a secondary manner in the production of rabbits circling to the left. However, the biochemical evidence does suggest that stimulation due to the accumulated ACh on one side of the cerebral cortex may be the primary factor in causing the animal to exhibit compulsive circling toward the contralateral side.

It occurred to the author that the number of observed turns per minute executed by the rabbits just prior to the removal of the brain parts for biochemical analysis might be a measure of the asymmetry in ACh concentration between the right and left cerebral cortex. Furthermore, if the above conclusion is correct, this should be true irrespective of the direction of the compulsive turning.

To test this idea, circus movements were produced in rabbits by injecting 0.1 mg./kg. DFP into the right common carotid artery. Nineteen "lefters" and seven "righters" are re-

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ported in this study. The number of turns executed by the rabbits were noted at different times. In the case of the 16 control animals, 0.1 ml./kg. water was injected instead of DFP. After the animal was observed for 20 minutes and had shown a single behavioral pattern, it was prepared for surgical excision of the desired brain areas. The tissue was processed and the ACh determined by a method previously described.[6]

In a given animal the free ACh content of the left cortex was subtracted from that found for the right cortex. This value in μg./gm. is expressed as $\triangle$ ACh $_{RC-LC}$ and is the unit used to plot against the turns per minute made by the animal just before it was sacrificed. In "lefters" $\triangle$ACh $_{RC-LC}$ was a positive value, because the right cortex had a higher ACh content than the left. In the case of the "righters," the left cortex had a higher ACh content and the difference in these two numbers was negative. The mean of the difference data between the right and left cortices of the 16 controls was + 0.03 μg./gm. Therefore, two assumptions were made: 1) the curve to describe the data passes through the origin, and 2) the curve is linear in the region studied. When the method of least squares was applied to the data, it was found that the slope of the curve for the "lefter" data was + 0.18, while that for the "righters" was –0.23. Therefore, a general equation to describe the relationship between the rate of turning, direction of turning, and the amount of asymmetry of ACh in the right and left cortices of rabbits induced to exhibit circus movements as a result of the injection of 0.1 mg./kg. DFP into the right common carotid artery is:

$$\pm D = kR$$

where D is $\triangle$ ACh $_{RC-LC}$ in μg./gm., k is 0.2, R is the rate of turning 20 minutes after the DFP injection ($R < 10$ t/min.), and a plus (+) sign indicates "lefters" while a minus (–) sign indicates "righters." No such correlation was found in the case of the caudate nuclei.

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Butyrylcholinesterase in human and ruminant nervous tissue

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THE ROLE of butyrylcholinesterase in central nervous tissue has been relatively unexplored, compared to acetylcholinesterase, because 1) we lack knowledge of the physiologic substrate for this enzyme (although acetylcholine has been suggested), and 2) its apparent absence from ruminant central nervous tissue makes it difficult to ascribe a fundamental function to this enzyme. Its absence from ruminant tissue has been based on the observation of Mendel, Mundell, and Rudney[1] that benzoylcholine is a specific substrate for the enzyme and no benzoylcholine hydrolysis is evident when this tissue is assayed with this substrate. Recently Hardwick[2] reported the isolation from bovine plasma of an enzyme which had properties similar to butyrylcholinesterase, except for its inability to hydrolyze benzoylcholine.

It was then decided to re-investigate the occurrence of this enzyme in ruminant brain.

Assays were performed in the conventional Warburg apparatus in the usual manner. Values are expressed as μmoles of substrate hydrolyzed per gram of wet tissue per hour. The differentiation of the two cholinesterases was made possible by the use of Mytelase (N',N'-bis-(2-diethylaminoethyl) oxamide bis-2-chlorobenzylchloride), which is a specific acetylcholinesterase inhibitor at the concentrations employed.

The results on human, bovine, and sheep brain white matter are shown in table 1.

Using butyrylcholine as a substrate, it can be seen that all three tissues exhibit activity to varying degrees and that this activity is only slightly inhibited by 5×10^{-7} *M* Mytelase. In addition, the ratios of butyryl- : acetylcholine activity are 2.4, 2.2, and 2.9 for human, bovine, and sheep white nervous tissue. This is in accord with the activity ratios found for butyrylcholinesterase in other tissues. In a double inhibitor experiment using Mytelase and 5×10^{-5} *M* eserine, the activity was completely abolished. It can also be seen that the ruminant tissue possessed no benzoylcholine-splitting properties. Further experiments on substrate concentration versus activity showed increasing activity of the enzyme with increasing substrate concentration. This also occurs

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TABLE 1

CHOLINESTERASES IN WHITE MATTER OF HUMAN, BOVINE, AND SHEEP BRAIN

	Human		Bovine		Sheep	
Substrate	*Q**	*Per Cent Inhibition*	*Q*	*Per Cent Inhibition*	*Q*	*Per Cent Inhibition*
AcCh•I, 1×10^{-2} *M*	24.2	–	11.7	–	16.5	–
" + inhibitor**	13.3	45.0	3.65	68.8	5.35	67.6
BuCh•Cl, 1×10^{-2} *M*	33.4	–	8.7	–	16.5	–
" + inhibitor**	32.2	3.6	8.1	7.0	15.7	5.0
BzCh•Cl, 1×10^{-2} *M*	8.6	–	0.0	–	0.0	–

*Q = μM substrate hydrolyzed/gm. wet tissue/hour

**Inhibitor: Mytelase, 5×10^{-7} *M*

Reaction mixture: Substrate, 1×10^{-2} *M*; buffer: 0.023 *M* $NaHCO_3$–0.154 *M* NaCl, pH 7.4; Atm.: 95% N_2 – 5% CO_2; hemogenate equivalent to 200 mg. wet tissue; total volume: 2.0 ml.

TABLE 2

OCCURRENCE OF BUTYRYLCHOLINESTERASE IN BRAIN WHITE MATTER IN VARIOUS SPECIES

Species	Butyryl-cholinesterase Q**	Butyrylcholinesterase + Inhibitor* Q	Per Cent Inhibition	Benzoyl-choline Q
Human	33.4	32.2	4	8.6
Bovine	8.7	8.1	7	0.0
Sheep	16.5	15.7	5	0.0
Eland	5.5	5.3	5	0.0
Kangaroo	16.7	14.7	12	0.0
Sita tunga	31.4	29.4	6	0.0
Swine	36.3	36.3	0	7.5
Horse	45.3	44.2	2	9.2

*Inhibitor: Mytelase, 5×10^{-7} *M*
**Q = μM substrate hydrolyzed/gm. tissue/hour
Reaction mixture: same as table 1

with the butyrylcholinesterase in serum, in contradistinction to acetylcholinesterase which has a definite substrate optimum.

Assays for butyrylcholinesterase in a few other species are presented in table 2. Enzymatic activity for butyrylcholine is evident and it can be seen that no benzoylcholine activity occurred in the marsupial or ruminant nervous tissue.

The data indicated that an enzyme is present in ruminant brain which is similar to the butyrylcholinesterase of human brain, except for its inability to hydrolyze benzoylcholine. Such a finding would imply that the enzyme may be ubiquitous in central nervous tissue and may serve a basic function in such tissue.

We are indebted to the Winthrop Laboratories for their generous gift of Mytelase.

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Effect of prolonged treatment with tranquilizing drugs upon two enzymes of the central nervous system

Winifred Ashby, Ph.D.

IT HAS BEEN REPORTED that carbonic anhydrase increases in the central nervous system in growing rats upon stimulation of the bone marrow by exposure to lowered atmospheric pressure and is reduced by increased pressure. Injections of bone marrow produced a more marked increase. Incidentally, it was found that chlorpromazine was as efficient a stimulant as bone marrow.[1,2] Further work is here reported using other tranquilizers and mature animals. Separate assays of the hippocampus have been made, as have parallel determinations of cholinesterase.

Batches of female rats and guinea pigs of approximately the same age were supplied by the National Institutes of Health. They were injected five times a week. Experimental and control animals were sacrificed alternately and the brain enzymes were assayed after brief freezing. Carbonic anhydrase was determined by a modification of the method of Philpots and Philpots, reproducing within 5 per cent. A titration method was used for cholinesterase. Statistical significance was determined by formulae from Rahn.[3]

In the saline injected controls changes were found in carbonic anhydrase activity both in the growing and in the mature animals. There were consistent increases in the cerebrum of four series studied. In the cerebellum and brainstem this consistency did not hold. The results of drug injections are considered with respect to absolute increase within the series and to per cent increase over the controls.

Bone marrow, chlorpromazine (0.44 mg./100 gm.), and reserpine (0.22 gamma/100 gm.) were injected for three and one-half and six and one-half weeks into growing rats. After three and one-half weeks there was increase in carbonic anhydrase in the cerebrum and brainstem but no significant change in the cerebellum. After six and one-half weeks increases in the controls had occurred in the cerebrum and brainstem but not in the cerebellum. In the injected animals carbonic anhydrase had increased in all areas. At this time the reserpine-injected animals showed the greatest content. In comparison with the controls, 17 injections of bone marrow and chlorpromazine gave statistically significant excess in the cerebrum and brainstem. Fifteen injections of reserpine showed statistically significant excess in the brainstem only. After 32 injections of bone marrow and 25 injections of reserpine, each series showed statistically significant excess over the controls in all areas, with the exception of the cerebrum in the bone marrow-treated animals which, although it had made further increase, had lost its lead.

In the next series the hippocampus was examined separately. Growing rats were injected with reserpine (10 gamma / 100 gm.) 19, 25, and 49 times. Twenty-five injections produced substantial increase in all areas, but after 49 injections there was a decrease in the cerebrum, hippocampus, and cerebellum. Eighteen injections of Frenquel (0.27 mg./100 gm.) produced increases in all areas; no further increase was shown with 31 injections, except in the brainstem. Increases in the hippocampus and brainstem were statistically significant at both periods of examination with both drugs.

Adult rats were treated with small doses of chlorpromazine (0.26 mg./gm.); 30 and 51 injections were given. After 30 injections the hippocampus was not examined separately and the only statistically significant increase found was in the cerebellum. After 51 injections

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there was significant excess over the controls in the hippocampus and brainstem, but not in the cerebellum and cerebrum.

Mature guinea pigs were treated with chlorpromazine (0.15 mg. per 100 gm.) and reserpine (6 gamma per 100 gm.), receiving 32 and 57 injections. After removal of the hippocampus, the cerebrum was divided into a frontal and occipital half and the brainstem was sectioned below the colliculi. After 32 injections there was a statistical excess over the controls in the frontal half of the cerebrum and in the hippocampus in both the chlorpromazine- and the reserpine-injected animals. After 67 injections with chlorpromazine the significant increase was in the occipital half of the cerebrum and in the lower and upper brainstem, while with reserpine it was in the lower brainstem. The hippocampus showed statistically significant increases in both series.

Cholinesterase did not show any increase in injected animals. Where any considerable difference between the controls and the injected animals occurred, it was a negative quantity.

A limited amount of data were collected, designed to disclose the part played by the bone marrow. Since carbonic anhydrase would seem to be of importance in facilitating the effect of carbon dioxide upon ionic exchange at the cell surface, it was assumed that the enzyme particles might be located at the cell surface and that the blood cell, with its high content of carbonic anhydrase, might deposit it on tissue cells activated to receive it. The data collected indicate no increase in corpuscles nor in the carbonic anhydrase content of the corpuscles to account for increases found in the central nervous system. Gross examination of the bone marrow at a break in the center of the hind leg, although it showed great individual variation, which negated a statistical significance, showed an average increased reddening in the injected animals as compared with the controls, which was greater in the beginning of each series so studied.

Increase in carbonic anhydrase of the kidney was found with injections of bone marrow, chlorpromazine, and reserpine.

The increase in carbonic anhydrase in the central nervous system, upon stimulation of the hemopoietic system by decreased oxygen and by bone marrow injection, suggests an endocrine function of the bone marrow with respect to carbonic anhydrase. If more meticulous testing should indicate an extension of the red bone marrow by the tranquilizing drugs, which might well be a compensatory process, this common increase in carbonic anhydrase with the tranquilizing drugs would be explained. But there still appears to be a specific effect upon the hippocampus and brainstem. In contrast to this effect, a pilot experiment run with Bogadine, a stimulating drug, disclosed a decrease in carbonic anhydrase in the hippocampus and brainstem, although some increase was shown in the cerebrum and cerebellum.

CONCLUSION

Superimposed upon a fluid state of carbonic anhydrase content of the central nervous system in the controls, the data presented would seem to clearly indicate that the tranquilizing drugs produce an increase in carbonic anhydrase in areas of the brain, which appears to culminate in the hippocampus and brainstem. In growing animals the effect is more diffuse than in adult animals. There has been shown an optimum point in the course of injections after which the carbonic anhydrase content deteriorates.

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Factor I and gamma-aminobutyric acid: A summary

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Inhibitory and excitatory effects of mammalian brain and spinal cord extracts on the generation of impulses by the stretch receptor neuron of the crayfish were first described by Florey.[1] The material responsible for the inhibitory effect was called "Factor I." The following is a summary of studies on this factor which have been made recently at the Montreal Neurological Institute.

Florey and Elliott[2] found certain interesting relationships between Factor I activity and neurotropic drugs. For instance, barbiturates, like Factor I, block the activity of the stretch receptor neuron, while substances which are convulsants in the crayfish, when applied in concentrations which did not affect the spontaneous activity of the neuron, prevented the inhibitory action of Factor I. A convenient method for assaying Factor I activity by means of the stretch receptor preparation was developed and it was found that most of the Factor I activity in brain is held in an "occult" or inactive form from which it could be released by heat, hypotonic medium, or dilute acid or alkali.

Using the assay method, Bazemore and Florey[3] set about isolating the factor. By alcohol extraction of acetone-dried beef brain, followed by the use of ion exchange resins and, finally, by fractional crystallization, the most active substance so far tested was obtained. This substance was identified as γ-aminobutyric acid (GAB) and authentic samples of this chemical were found to have the same activity. Among a large number of other amino acids and other compounds of physiologic interest which have been tested, obvious Factor I activity has been observed only with γ-amino-β-hydroxybutyric acid which is about half as active as GAB, with β-alanine and δ-aminovaleric acid which were about one-twentieth as active, and with α,γ-diaminobutyric acid which is about one-sixtieth as active. Carnitine and β-hydroxybutyric acid and α-aminobutyric acid seemed inactive. Glutamic acid gives excitatory and inhibitory effects with some, but not all, stretch receptor preparations and, very rarely, has shown inhibitory effects nearly as strong as with GAB. The presence of considerable amounts of GAB in brain was discovered in 1950 by Awapara, Landua, Fuerst, and Seale,[4] Roberts and Frankel,[5] and Udenfriend.[6] The amounts found to be present could apparently account for all the Factor I activity observed but, so far, we have no real evidence that GAB is the only substance in brain which is strongly active as Factor I.

Studies by Florey and McLennan[7,8] and Florey,[9] with partially purified extracts containing Factor I, have indicated that the factor strongly affects the activity of various mammalian and invertebrate nervous structures. Hayashi and Nagai[10] have reported that γ-amino-β-hydroxybutyric acid, applied to the cortex or injected into the carotid artery or the cerebrospinal fluid, inhibits electrically or chemically induced convulsions in dogs. This substance is not known to be present in brain. Gamma-aminobutyric acid was also inhibitory in low concentrations but in higher concentrations it became a "stimulant."

Recently, Iwama and Jasper[11] have found that GAB, topically applied to the exposed cortex of cats, causes reversible depression of the surface negative phase of sensory evoked potentials, increases after-discharges, changes the larger amplitude waves of the recruiting response from surface negative to positive, increases spontaneous electrical activity, and reverses the predominant polarity of "spindle bursts." The surface negative dendritic re-

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sponse to local cortical stimulation is abolished, the effect being narrowly localized to the site of application. No effect on evoked potentials occurred when the substance was applied about 1 mm. beneath the surface of the cortex. Very similar observations have been made by Purpura and Grundfest.

These results, together with those of Killam and Bain[12,13] showing that hydrazide intoxication is accompanied by a fall in the γ-aminobutyric acid level of the mammalian brain concomitant with the convulsive activity produced by the hydrazides, strongly suggest that GAB is concerned in some aspects of the regulation of the physiologic activity in the brain and other nervous structures.

If this is so, we must expect the tissue to have machinery for the production, storage, release, and final disposition of the active substance. These aspects are being studied.

It is well known that brain tissue contains a pyridoxal phosphate-dependent decarboxylase which can produce GAB from glutamate. On incubation in medium containing added glutamate, the GAB content of slices of cerebral cortex increases very considerably. This increase is dependent upon the concentration of glutamate.

As was mentioned previously, GAB in brain tissue seems to be nearly all in a bound or storage form.

Neither brain suspensions nor slices destroy GAB at any appreciable rate when it is added to the medium. Bessman, Rossen, and Layne[14] and Roberts and Bregoff[15] have shown that brain preparations can cause GAB to undergo a transamination reaction with *a*-ketoglutarate. This, however, occurs at an appreciable, but still low, rate only when relatively high concentrations of both reactants are present.

When brain slices are immersed in medium containing GAB, they rapidly absorb large amounts of the GAB. The concentration of GAB in the slices can increase from about 3 to 25 μmoles per gm., while the amount of free GAB in the medium is decreased. It is found that the ratio of the concentration of GAB in the slice to that in the medium increases strongly as the concentration in the medium is decreased. In medium containing 0.5 μmoles per ml., for example, the slices contain about 17 μmoles per gm. If a physiologically active concentration of free GAB were brought in contact with the tissue, the tissue would be able to absorb it virtually all into the bound form. This absorption depends on tissue structure since it does not occur with disintegrated tissue. It does not occur with diaphragm muscle or with slices of liver or kidney. This absorption could then serve as a mechanism by which free active GAB is disposed of physiologically.

We have not yet had opportunity to seek for conditions of physiologic significance which could cause the release of active GAB from the bound form.

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Cerebral blood flow in epileptic children during the interseizure period

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SINCE THE DEMONSTRATION by Cooper[1] in 1836 that convulsions could be produced experimentally by restricting the blood flow to the brain, the possible role of abnormalities of the cerebral circulation in epilepsy has been studied extensively. A once widely held theory that cerebral vessels of the epileptic are subject to generalized vasospasm, which produced the seizure by causing cerebral anemia,[2] has been largely disproved,[3,4] while considerable evidence exists in support of the possibility that local alterations of cerebral vessels may be present in many seizure-prone individuals.[5–8] However, quantitative measurements of over-all cerebral blood flow in adult epileptics during the interseizure period, which might be affected by local vascular abnormalities, have not revealed any significant difference from that in normal individuals.[9,10] The demonstration that the cerebral blood flow in children is nearly twice that of adults[11] suggested the possibility that a difference, particularly if small in proportion to total cerebral blood flow and therefore obscured by errors of the method, might be more readily detected in the pre-adolescent age group. Therefore, values for cerebral blood flow were obtained in a group of epileptic children, using a modification of the nitrous oxide method. In a preliminary report, no significant difference was found between the mean value in this group and that of normal children.[12] However, more recently the number of determinations in both normal and epileptic children has been increased. A statistical analysis of the data of the enlarged series constitutes the basis for the present report.

METHOD

The cerebral blood flow (CBF) of 12 children subject to a variety of epileptiform seizures was determined by means of a modification of the nitrous oxide technic.[11] Similar determinations were made in 11 normal children previously reported,[11] with the addition of two studies, one in a ten year old girl and one in an eight year old boy.

The character and total number of attacks in the epileptic children together with the electroencephalographic findings are listed in table 1. Neurologic examinations were negative in all instances, as were skull roentgenograms, and no evidence of metabolic disease was found in routine urinalyses and blood chemistry determinations. All but four of the children were considered mentally normal on the basis of developmental history and school adjustment. Psychologic testing done on the four in whom some question had arisen revealed that three (cases 3, 9, and 12) were functioning at a dull normal level, while the fourth (case 7) had above average mental ability. In the pneumoencephalogram done in the one child (case 12) with intractable seizures, atrophy in the region of the insula was found on the right side. A history of head trauma resulting in a concussion and skull fracture five years prior to the onset of his attacks was obtained on one child (case 1). In none of the near relatives was there a history of epilepsy. Two children could not be taken off anticonvulsant medication for the cerebral blood flow determination without serious aggravation of their seizure status. Therefore, although the data from their studies are given in tables 2 and 3, these were not included in

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TABLE 1
CLINICAL DESCRIPTIONS OF 12 EPILEPTIC CHILDREN

Subject	*Sex*	*Character of Attacks*	*Electroencephalogram*	*Total Number of Attacks*
1.	M	Generalized	Abnormal	5
2.	M	Generalized and petit mal	Borderline	< 10
3.	M	Generalized	Normal	10
4.	M	Generalized	Normal	5
5.	M	Generalized	Abnormal	> 50
6.	F	Petit mal	3/sec. spike and wave	> 50
7.	M	Petit mal	3/sec. spike and wave	> 50
8.	M	Generalized and petit mal	Abnormal	> 50
9.	M	Akinetic	Abnormal	> 20
10.	F	Akinetic and psychomotor	Normal	> 50
11.	M	Generalized	Abnormal	> 20
12.	M	Focal motor and generalized	Abnormal	> 50

the statistical analysis. One of these (case 12) was maintained on 0.2 gm. of phenobarbital per day, in spite of which he continued to have some focal twitching of the left face several times per day, and the other (case 11) continued to receive 0.15 gm. of diphenylhydantoin and 0.06 gm. of phenobarbital per day, without which generalized seizures were precipitated. The remaining ten had been off all medication for at least three days prior to the study. Except for two children (cases 6 and 7) with pure petit mal,* who had had two or three attacks each on the day prior to the study, all had been free of seizures for a week or more prior to the cerebral blood flow determination.

Mean arterial blood pressure (MABP) was measured by means of a damped mercury manometer connected to the femoral arterial needle. Blood oxygen and carbon dioxide content were determined by the technic of Van Slyke and Neill,[13] and cerebral oxygen consumption ($CMRO_2$) and cerebral vascular resistance (CVR) were calculated in the usual manner.[14] Hemoglobin concentration was measured as cyanomethemoglobin[15] in a Klett photoelectric colorimeter, using the copper sulphate standard.[16] Blood pH was measured anerobically at room temperature in a Cambridge pH meter and was corrected to 37° C. by means of the factors of Rosenthal.[17]

*Momentary absence of consciousness without a motor component and having three per second spike and wave discharges in electroencephalogram.

RESULTS

The data obtained in the individual studies in epileptic children and the mean values for the normal series are given in tables 2 and 3. With cases 11 and 12 omitted from the statistical analysis because of their anticonvulsant medication, it can be seen that a statistically significant difference exists between the mean value of 106 cc./100 gm./min. for CBF in the normal children, and that of 92 cc./100 gm./min. in the ten children with epilepsy ($p < 0.05$). Likewise the mean CVR of 0.91 mm. Hg/cc./100 gm./min. in the latter group is significantly greater than that of 0.76 mm. Hg/cc./100 gm./min. for the normal series ($p < 0.05$), the MABP being the same for both series. The lower $CMRO_2$ of 4.72 cc./100 gm./min. in the seizure group, which compared with 5.22 cc./100 gm./min. in the normal children, is not of statistical significance ($p < 0.1$). The mean arteriovenous oxygen difference and the cerebral respiratory quotient were comparable for both groups and no significant differences were found among the blood constituents.

The ten epileptic children off medication may be divided into two groups. One might be designated as those with well controlled epilepsy and constitute the first four children in table 1. Each of these had had a total of ten or less seizures and could be kept seizure-free for periods of a year or more with proper medication. A second group, the next six cases in table 1, could be considered poorly controlled, since each child in this group had had

Table 2
Blood Constituents

Subject	Hemoglobin gm. %	O_2 Content Vol. %		CO_2 Content Vol. %		pH		CO_2 Tension mm. Hg	
		Arterial	Jug. Venous	Arterial	Jug. Venous	Arterial	Jug. Venous	Arterial	Jug. Venous
TWELVE EPILEPTIC CHILDREN									
1.	12.7	17.03	11.66	42.30	48.24	7.38	7.34	37	45
2.	12.8	16.27	12.05	47.92	53.69	7.38	7.33	42	51
3.	12.9	16.55	12.49	43.48	47.99	7.37	7.32	39	47
4.	12.6	15.31	10.52	49.85	53.82	7.36	7.32	45	53
5.	14.2	18.76	11.52	47.62	54.93	7.35	7.32	45	55
6.	10.4	13.22	8.86	49.23	52.27	7.37	7.31	42	51
7.	14.1	18.75	11.49	45.78	52.22	7.40	7.33	39	51
8.	13.0	17.00	10.56	45.99	52.20	7.38	7.35	40	48
9	11.8	15.04	11.47	50.03	53.16	7.38	7.34	43	49
10.	13.0	15.81	9.86	46.27	51.82	7.38	7.33	42	49
11.*	10.4	13.58	8.24	48.96	54.54	7.38	7.35	41	49
12.*	14.8	16.68	11.40	49.71	56.02	7.41	7.37	41	49
Mean	12.80	16.37	11.05	46.85	52.03	7.38	7.33	41.4	49.9
Standard Error	±0.34	±0.53	±0.35	±0.82	±0.72	±0.004	±0.003	±0.8	±0.9
ELEVEN NORMAL CHILDREN									
Mean	12.31	15.62	10.72	46.12	51.06	7.38	7.34	40.1	48.1
Standard Error	±0.22	±0.28	±0.25	±0.70	±0.72	±0.01	±0.01	±0.9	±2.5
p value	>0.2	>0.2	>0.2	>0.5	>0.3	—	>0.4	>0.2	>0.5

*Not included in statistical analysis because of anticonvulsant medication.

20 or more seizures and could be kept seizure-free only for periods much shorter than a year. This group includes the two children with petit mal, whose attacks had numbered 20 to 50 per day for many consecutive weeks in certain periods in the past. With the series so subdivided, it can be seen from table 4 that the mean value for CBF in the children with mild seizure proneness is comparable to that of the normal series. That for the severe epileptics, 82 cc./100 gm./min., is significantly lower than both the normal mean and the mean of the mild epileptics, the p value being < 0.01 and < 0.05, respectively. Likewise, mean CVR of 1.02 mm. Hg/cc./100 gm./min. for the severe epileptics is significantly higher than 0.76 mm. Hg/cc./100 gm./min. for the normal children and also higher than 0.70 mm. Hg/cc./100 gm./min. for the mild epileptics, the p value being < 0.02 in both comparisons. No significant differences were found in the $CMRO_2$ nor in any of the blood constituents on comparing the severe epileptics with the mild epileptic or with the normal children.

The two epileptic children (cases 11 and 12) are reported as instances of such severe seizure proneness that medication could not be withdrawn without precipitating a severe exacerbation of attacks. These two, not included in the statistical analysis for this reason, are of interest because their values for CBF, 77 and 63 cc./100 gm./min., respectively, are among the lowest of the entire group studied. Also the CVR of 1.2 and 1.3 mm. Hg/cc./100 gm./min., respectively, exceed those for any of the other epileptic children. Since barbiturates have been shown to produce no effect on CBF in sedative doses,[18] or even to result in an increase in CBF if given in larger doses,[19] it seems unlikely that these extreme values for CBF and CVR

Table 3
Cerebral Circulatory Function
TWELVE EPILEPTIC CHILDREN

Subject	Sex	Age Years	MABP mm. Hg.	CBF cc./100 gm./min.	$CMRO_2$ cc./100 gm./min.	$(A\text{-}V)O_2$ Vol. %	CVR mm. Hg. cc./100 gm./min.	Cerebral R.Q.
1.	M	11	—	91	4.9	5.37	—	1.10
2.	M	7	80	111	4.7	4.22	.7	1.36
3.	M	5	74	107	4.3	4.06	.7	1.11
4.	M	7	79	109	5.2	4.79	.7	.83
5.	M	11	103	91	6.6	7.24	1.1	1.01
6.	F	7	77	99	4.4	4.36	.8	.70
7.	M	8	74	61	4.4	7.26	1.2	.89
8.	M	9	80	77	5.0	6.44	1.1	.95
9.	M	10	76	99	3.5	3.57	.8	.88
10.	F	10	80	71	4.2	5.95	1.1	1.03
11.*	M	3	89	77	4.1	5.34	1.2	1.04
12.*	M	8	85	63	3.9	6.28	1.3	1.00
Mean		8.5	80.3	91.6	4.72	5.33	.91	.99
Standard Error		±0.6	±2.8	±5.4	±0.26	±0.72	±0.07	±0.06
ELEVEN NORMAL CHILDREN								
Mean		6.6	81.5	106.3	5.22	4.91	.76	1.01
Standard Error		±0.9	±1.6	±3.0	±0.20	±0.10	±0.02	±0.04
p value		>0.1	>0.6	<0.05	>0.1	>0.9	<0.05	>0.8

*Not included in statistical analysis because of anticonvulsant medication.

are due to the phenobarbital. With respect to the possible effect of diphenylhydantoin, we have conducted a preliminary study, the results of which are given in table 3. In a group of four epileptic children studied before and again a week after being placed on the usual anticonvulsant doses of this drug (120 to 200 mg. per day), it can be seen that there is an increase in the mean CBF from 85 cc./100 gm./min. to 102 cc./100 gm./min., the p value being < 0.01. Therefore, it would also seem unlikely that the low value for CBF in case 11 is due to the effect of diphenylhydantoin.

DISCUSSION

The finding of a significantly lower mean CBF in a group of epileptic children in the interseizure period is perhaps surprising, in view of the reports of Gibbs[9] and of Grant and co-workers[10] that no such difference exists in adults. While the negative findings of the former might be explained on the basis of errors inherent in the dye injection technic, the discrepancy with the latter is less apparent. It is noteworthy, however, that all of Grant's patients were subject to generalized seizures. Therefore, our patients, which included children subject to petit mal, akinetic, and psychomotor attacks in addition to generalized seizures, are not entirely comparable. Indeed, if the four children in our series who had generalized seizures only are considered separately, their mean CBF of 100 cc./100 gm./min. is not significantly lower than the mean normal of 106 cc./100 gm./min. ($p > 0.1$). Since three of the four children with grand mal attacks alone were also considered to have mild seizure proneness, it is possible that the lack of a lowering of CBF is related to this factor, rather than to the character of attacks. In support of this view is the absence of a

TABLE 4

COMPARISON OF CBF IN CHILDREN WITH WELL CONTROLLED SEIZURES VS. THOSE POORLY CONTROLLED

	CBF cc./100 gm./min.	*$CMRO_2$ cc./100 gm./min.*	*CVR, mm. Hg/ cc./100 gm./min.*
Well controlled (4 cases). Total number of attacks < 10. Free of seizures for a year or more	108	4.8	.7
Poor or only fair control (4 cases). Total number of attacks < 50, having attacks more often than once per month	82*	5.1	1.1**

* $p < .05$
** $p < .01$

significant difference between the mean CBF of those with pure grand mal epilepsy of 100 cc./100 gm./min. and 86 cc./100 gm./min. of the remainder of children with petit mal, akinetic, and mixed type of seizures, $p > 0.2$.

The results of this study are consistent with the hypothesis that there are functional alterations of cerebral vasculature of epileptics, such as have been observed directly during craniotomy.[21] They also might be expected in epileptic individuals with local cerebral scars, the histology of which have been described.[5–7] Penfield[8] also has described focal atrophic scars which he considered epileptogenic because of the electrical hyperexcitability of neural tissue on their periphery, the central portions being avascular and having a low oxygen tension. If such lesions were present in the individuals studied here, the oxygen consumption of the brain might also be depressed, in addition to the lowering of CBF. However, the observation that functional alteration of the brain under moderate hypoxia is observed in the absence of a fall in $CMRO_2$[22] indicates that this need not necessarily be the case. It is of interest that in case 12, who suffered focal attacks and had evidence of localized cerebral atrophy in his pneumoencephalogram, the CBF was among the lowest of all the epileptic children. This would suggest that functional impairment of CBF may take place over a much greater portion of brain substance than is evident by pneumoencephalography from a grossly demonstrable region of atrophy.

Carbon dioxide content of arterial and jugular venous blood was found by Gibbs[23] to vary over a wider range in epileptics in the interseizure period than in normal individuals. Those prone to petit mal attacks were found to have a low blood carbon dioxide content, while those with generalized seizures had elevated values. These findings are not confirmed in the present study. Not only is there no tendency for a grouping of the two seizure types at either extremes, but the variance of both arterial and jugular venous carbon dioxide content of the epileptics is not significantly

TABLE 5

EFFECT OF DIPHENYLHYDANTOIN ON CEREBRAL CIRCULATORY FUNCTIONS IN FOUR EPILEPTIC CHILDREN

Subject	*CBF cc./100 gm./min.*		*$CMRO_2$ cc./100 gm./min.*		*CVR, mm. Hg cc./100 gm./min.*	
	C	*E*	*C*	*E*	*C*	*E*
R.A. (case 8)	77	94	5.0	5.2	1.1	.9
W.T.	105	117	6.1	5.4	.7	.6
D.S. (case 1)	91	108	4.9	6.0	–	.8
B.N.	65	90	3.4	4.2	–	.8
Mean	84.5	102.3*	4.85	5.20	.90	.77

* $p < 0.01$ as determined by the method of paired comparisons.

greater than that of the normal children, the p value being > 0.2 in each instance. Also, the mean carbon dioxide tension of arterial and jugular venous blood, which may be more directly related to alterations in CBF,[22] is not significantly different from normal.

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N-mythelated derivatives of barbituric acid, hydantoin and oxazolidinedione used in the treatment of epilepsy

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THE DRUGS used most extensively at the present time in the treatment of epilepsy are derivatives of the three related heterocyclic structures, barbituric acid, hydantoin, and 2,4-oxazolidinedione. Some workers in the field have been of the opinion that substitution of a methyl group on nitrogen in these structures specifically enhances antiepileptic activity relative to undesirable actions. There are now five such methylated compounds commercially available in this country for use in epilepsy. Two are derivatives of barbituric acid: mephobarbital (Mebaral), which is the N-methyl derivative of phenobarbital, and metharbital (Gemonil), which is the N-methyl derivative of barbital. One is a derivative of hydantoin: Mesantoin, which is the N-methyl derivative of Nirvanol. Two are derivatives of 2,4-oxazolidinedione: trimethadione (Tridione), which is the N-methyl derivative of 5,5-dimethyl-2,4-oxazolidinedione (to be designated DMO), and paramethadione (Paradione), which is the N-methyl derivative of 5-ethyl-5-methyl-2,4-oxazolidinedione (to be designated EMO).

All of these methylated compounds are demethylated in the body. Mephobarbital is converted to phenobarbital.[1,2] Metharbital is converted to barbital.[1,3] Mesantoin is converted to Nirvanol.[4,5] Trimethadione is converted to DMO.[6,7] Paramethadione is converted to EMO.[8] Ultraviolet spectrophotometric methods have been devised for the analytic determination of the demethylated products in plasma. With the hydantoins and oxazolidinediones only the non-methylated compounds can be determined, but with the barbituric acids the methylated and non-methylated compounds can be determined simultaneously. These analytic methods have made it possible to study quantitatively the course of the demethylation reaction and to estimate the extent of the reaction. When single doses of the methylated drugs are administered to dogs, demethylation requires one to two days for completion. However, the products of demethylation are eliminated very slowly and they persist in the plasma long after the parent drugs have disappeared. Estimates of the extent of demethylation based on the plasma concentrations of the products indicate that all of the methylated drugs mentioned above are converted almost completely by the dog to the corresponding non-methylated compounds.

Although demethylation proceeds at a slow rate, the demethylated product is eliminated at an even slower rate. Accordingly, administration of a methylated drug in a chronic schedule will result in more extensive accumulation of the demethylated product than of the original drug. The present report presents measurements of the concentrations of demethylated products in the plasma of human subjects receiving the methylated drugs in chronic dosage schedule, concluding with an attempt to assess the importance of the demethylated products in the therapeutic and toxic effects seen after administration of the methylated drugs.

MEPHOBARBITAL

Figure 1 shows the concentrations of unchanged mephobarbital and of phenobarbital

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in the plasma of a man who received mephobarbital for 14 days. Phenobarbital accumulated in concentrations much higher than those of the unchanged methylated drug. Similar results were obtained in two other subjects who received mephobarbital in the same dosage schedule. In none of the three subjects was the highest observed mephobarbital concentration as much as 30 per cent of the highest phenobarbital concentration.

For these three subjects estimates have been made of the amount of the total administered dose of mephobarbital that can be accounted for on the day after dosage was discontinued. The amounts of mephobarbital and phenobarbital present in the body at that time were estimated from the plasma concentrations, on the assumption that the apparent volumes of distribution have the same values as those previously measured in the dog.[2] The amount of phenobarbital lost by excretion and chemical destruction prior to that time was estimated from the rate of decline of the plasma concentration in each subject after discontinuation of dosage. The assumption was made that the rate of elimination of phenobarbital is proportional to the plasma concentration present at the time. This has been shown to be approximately true.[9] Only traces of unchanged mephobarbital appear in the urine and this was neglected. In all of the three subjects the sum of the amount of mephobarbital in the body, the amount of phenobarbital in the body, and the amount of phenobarbital lost from the body was equivalent to 50 to 60 per cent of the administered dose of mephobarbital. These estimates are rough, but it seems clear that a large part of the dose of mephobarbital is unaccounted for. It is either unabsorbed or is destroyed by reactions other than demethylation. In the dog it has been shown that intravenously administered mephobarbital is almost completely converted to phenobarbital, with little, if any, undergoing any other reactions.[2] Also in the dog, orally administered mephobarbital gives plasma concentrations of phenobarbital about half those resulting from orally administered phenobarbital in equivalent dosage. Mephobarbital is evidently incompletely absorbed by the dog, and there is little doubt that the amount unaccounted for in these three human subjects

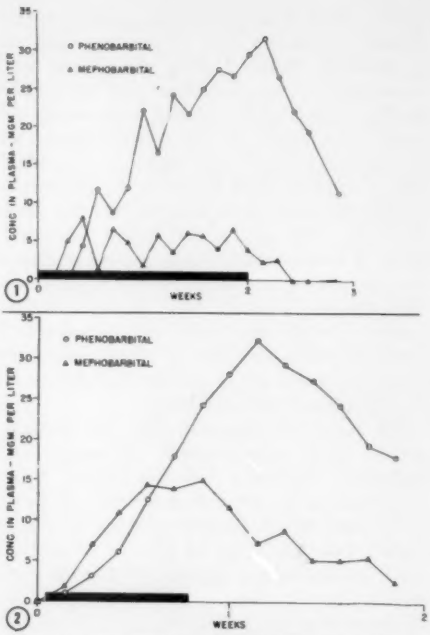

FIG. 1. Concentrations of phenobarbital and of unchanged mephobarbital in the plasma of a 72 kg. man who received 0.2 gm. of mephobarbital three times a day for 14 days. The bar indicates the period in which the drug was administered.
FIG. 2. Concentrations of phenobarbital and of unchanged mephobarbital in the plasma of a 58 kg. man who received 0.2 gm. of mephobarbital three times a day for five days. The bar indicates the period in which the drug was administered.

was unabsorbed. The doses of mephobarbital usually recommended are about double those of phenobarbital. This would suggest that incomplete absorption of mephobarbital, such as was found in these three men, is the typical pattern. The poor absorption of mephobarbital may be due to low solubility in water. The solubility of mephobarbital in water is only about one-twentieth that of phenobarbital.

In one other human subject the plasma concentrations of unchanged mephobarbital were considerably higher than in the three subjects described above. Depression was so pronounced in this subject that the drug was discontinued after ten days. The experiment was repeated in the same man a year later. On this occasion dosage was discontinued after five days because of depression. The

plasma level of mephobarbital reached in the second experiment was almost the same as in the first. Figure 2 shows the plasma concentrations of mephobarbital and of phenobarbital found in the second experiment. In both experiments on this subject nearly all of the administered dose could be accounted for as mephobarbital in the body, phenobarbital in the body, and phenobarbital lost from the body. This subject had higher concentrations of mephobarbital than the other three, not because of any defect in his capacity to demethylate the drug, but because he absorbed it much more completely. Mephobarbital was being absorbed at a rate exceeding that at which it could be demethylated, and it accumulated at high levels.

No reason is apparent why one subject should absorb mephobarbital completely and others to only about half that extent. What proportion of the population absorbs the drug completely, like the subject of figure 2, is not known. On the basis of plasma concentrations, mephobarbital appears to be one and one-half to two times as active a depressant of the central nervous system as is phenobarbital in the dog.[2] Differences in the extent of absorption of mephobarbital might be expected to result in large differences in the depressant effects. Variations from individual to individual in completeness of absorption, and consequently in the intensity of depressant effects, would be an undesirable feature of the use of mephobarbital.

METHARBITAL

Figure 3 shows the plasma concentrations of unchanged metharbital and of the barbital produced from it in a subject who received metharbital for 14 days. Similar results were obtained in three other subjects who were given the drug in the same schedule. In none of the four subjects did the concentration of unchanged methylated drug ever exceed 20 per cent of the highest concentration to which barbital finally accumulated.

MESANTOIN

The analytic method that has been developed allows the determination only of the product of demethylation, Nirvanol, but not of the original methylated drug. Figure 4 shows the accumulation of Nirvanol in the plasma of a man who received Mesantoin for 14 days. If the apparent volume of distribution of Nirvanol is the same as in the dog,[5] the amount of Nirvanol remaining in the body at the time of the highest concentration is equivalent to about a third of the administered dose of Mesantoin. If allowance is made for the loss of Nirvanol before that time, it may be inferred that the concentration of unchanged Mesantoin present at any time must be much lower than the concentration to which Nirvanol accumulates in a chronic dosage schedule.

TRIMETHADIONE

Figure 5 shows the concentrations of demethylated trimethadione (DMO) in the plasma of a man who received trimethadione for six days. The metabolic product, but not the original drug, can be measured with the analytic method used. The concentration of DMO in the plasma continued to rise throughout the period of administration and during the day after dosage had been discontinued. Thereafter, the elimination of DMO proceeded with remarkable slowness. The proportion disappearing daily was about 6 per cent. DMO is among the most persistent of all organic compounds hitherto studied.

If the apparent volume of distribution of DMO is the same in man as in the dog,[7] the total amount of DMO in the body of this subject at the time of the highest concentration would correspond to about 85 per cent of the total dose of trimethadione received. The amount of DMO estimated to have been lost before the attainment of the peak concentration almost exactly accounts for the remainder of the dose. Accordingly, it can be concluded that trimethadione was almost completely converted to DMO by this man, and that after several days of dosage the amount of unchanged trimethadione in the body at any time could not have been more than a small fraction of the amount of DMO present.

Four years after this experiment, the same man was given the same molar dose of DMO itself in the same schedule. The plasma concentrations of DMO found in this experiment are also shown in figure 5. For any value of DMO concentration during the period of ac-

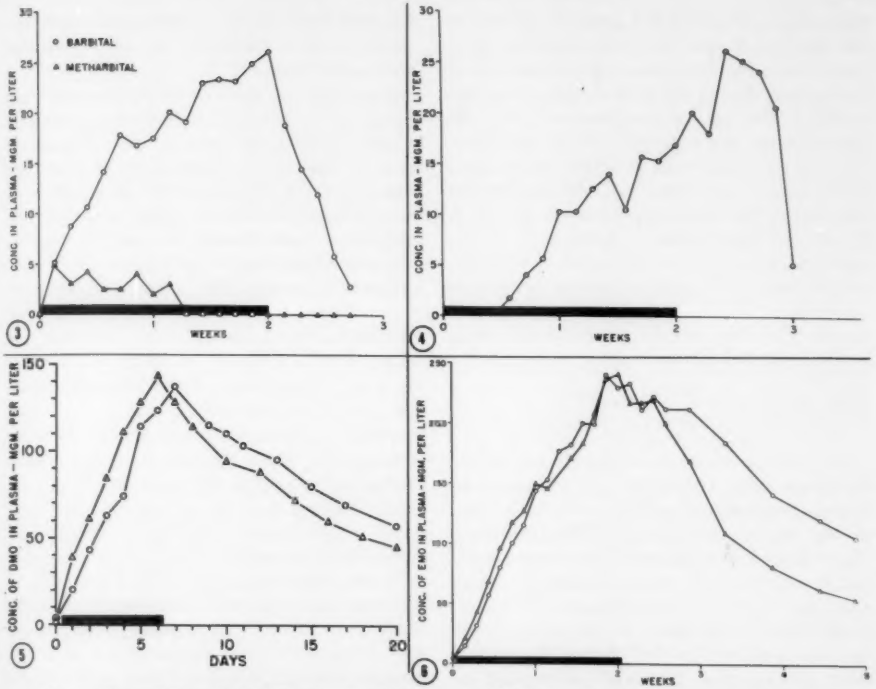
BARBITAL
METHARBITAL
CONC. IN PLASMA - MGM. PER LITER
WEEKS
CONC. OF DMO IN PLASMA - MGM. PER LITER
DAYS
CONC. OF EMO IN PLASMA - MGM. PER LITER

FIG. 3. Concentrations of barbital and of unchanged metharbital in the plasma of a 49 kg. man who received 0.1 gm. of metharbital three times a day for 14 days. The bar indicates the period in which the drug was administered.

FIG. 4. Concentrations of Nirvanol in the plasma of a 64 kg. man who received 0.1 gm. of Mesantoin three times a day for 14 days. The bar indicates the period in which the drug was administered.

FIG. 5. Concentrations of 5,5-dimethyl-2,4-oxazolidinedione (DMO) in the plasma of a 75 kg. man when he received 0.3 gm. of trimethadione three times a day for six days (points designated by circles) and in the plasma of the same man when he received the same molar dose of DMO itself in the same schedule four years later (points designated by triangles). The bar indicates the period in which the drug was administered.

FIG. 6. Concentrations of 5-ethyl-5-methyl-2,4-oxazolidinedione (EMO) in the plasma of two men who received 0.3 gm. of paramethadione three times a day for 14 days. The bar indicates the period in which the drug was administered. The men weighed 61 kg. (triangles) and 92 kg. (circles), respectively. (From J. Pharmacol. & Exper. Therap. 113:178, 1955).

cumulation, the level was attained about a day sooner if DMO was administered than if trimethadione was administered. This difference may be attributed to the time required for demethylation of trimethadione. Otherwise there is a very close correspondence between the concentrations found in the two experiments, indicating that trimethadione is almost completely converted to DMO by man.

The experiments of figure 5 were not continued for a time long enough for equilibrium to be attained, that is, for DMO to reach a concentration where its rate of elimination would equal its rate of production or intake. It can be calculated that dosage for an indefinite time on the same schedule in this subject would have led to accumulation of DMO in a concentration about three times as high as those attained in these experiments. The concentrations of DMO found in the plasma of patients receiving the usual doses of trimethadione or equivalent amounts of DMO have ranged between 200 and 950 mg. per l. The patient with the concentration of 950 mg. per l. showed no evidence of toxic effect.

PARAMETHADIONE

Paramethadione is the homologue of trimethadione and is handled similarly by the body. In figure 6 are shown the concentra-

tions of the demethylated product (EMO) in the plasma of two men who received paramethadione for 14 days. EMO continued to accumulate during the period of drug administration. The rate of disappearance of EMO from plasma, like that of DMO, is very slow. Calculation of the yield of EMO by the same method described above for DMO leads to the conclusion that paramethadione was practically completely converted to EMO in these two subjects. It can also be concluded that chronic administration of paramethadione results in amounts of EMO in the body many times greater than the amount of unchanged paramethadione that can be present at any time.

ROLE OF DEMETHYLATED PRODUCTS IN THERAPEUTIC AND TOXIC EFFECTS

All five of these N-methyl derivatives of barbituric acid, hydantoin, and oxazolidinedione are extensively, perhaps essentially completely, demethylated by man. When the methylated drugs are administered in chronic schedule, there is in every instance direct or indirect evidence to indicate that the product of demethylation accumulates in the body in an amount much greater than that of the original drug. The question then arises as to what extent the demethylated products may be responsible for the therapeutic and toxic effects seen after administration of the methylated drugs.

This is a question that can be answered only by trial in human epilepsy, not by tests in laboratory animals. It is true that the most valuable antiepileptic agents introduced in recent years have been discovered through laboratory tests, but there is no assurance that any laboratory procedure is such a satisfactory counterpart of any type of human seizure that it can be relied upon to disclose activity in close correlation with activity in that type of human seizure. Furthermore, laboratory testing has usually been done with acute dosage rather than with the chronic schedule used clinically. If a test is carried out on an animal shortly after administration of a single dose of one of these methylated drugs, there will be present in the body at that time a higher proportion of unchanged drug relative to demethylated product than will be present in a patient on a chronic dosage schedule. Thus, differences between the methylated and non-methylated drugs as observed in anticonvulsant tests in laboratory animals are of dubious significance.

Some of the demethylated products are known to be clinically effective antiepileptic agents, but little is known of the efficacy of some of the others. Best recognized is the effectiveness of phenobarbital, the product of demethylation of mephobarbital. Barbital, the product of demethylation of metharbital, is generally considered to be ineffective in epilepsy and it has had little, if any, trial in recent years. However, there have been reports of effective control of seizures with barbital.[10,11] Nirvanol, the product of demethylation of Mesantoin, was shown to be a clinically effective antiepileptic agent.[12,13] It was abandoned for this use on account of toxicity, not because of inactivity. There has, as yet, been no adequate clinical trial of the non-methylated oxazolidinediones, but in one case of petit mal epilepsy now being treated with DMO by Dr. Harrie R. Chamberlin, the drug has appeared to be effective. Thus, all of the demethylated products under consideration are either definitely known to be effective or can be reasonably presumed to have some degree of effectiveness in human epilepsy. There can be little doubt that they must play at least some part in the therapeutic effects seen after administration of the methylated drugs. Unless the methylated drug possesses some specific type of activity lacking in the demethylated product, or unless its activity is very much higher than that of the product, the relatively small amount of unchanged drug could not be responsible even for a major part of the therapeutic effect. There is at present no satisfactory evidence that the methyl group on nitrogen greatly enhances activity or confers qualitatively different activity in human epilepsy. It is true that there have been some reports that mephobarbital is qualitatively superior to phenobarbital in the control of some types of seizures, but the evidence is not convincing. Evaluation of the therapeutic role of the unchanged form of a methylated drug would require a statistically controlled comparison of the methylated drug with the corresponding non-methylated compound in regard to their effectiveness in suppressing human seizures. No study adequately designed to satisfy the

statistical requirements of such a controlled comparison has as yet been carried out for any one of the pairs of compounds that have been considered here. There is at present no convincing evidence that the therapeutic effects of this group of methylated drugs cannot be accounted for, in large part at least, as the actions of the demethylated products. This is not to imply that the methylated compounds are inactive. Even if they were somewhat more active than the corresponding non-methylated compounds, they might, nevertheless, play a minor therapeutic role because of the relatively small amounts in which they are present in the body.

The demethylated products undoubtedly make an important contribution to the undesirable effects as well as to the therapeutic effects when methylated drugs are administered. No statistically adequate comparison has been made of any of these pairs of drugs in regard to their undesirable effects. This is particularly true with respect to drowsiness and other psychic effects. Such effects are ill-defined and subject to influence by suggestion, and their evaluation has amounted to little more than vague impressions on the part of patient and physician. For instance, claims have been made that mephobarbital in therapeutically effective dosage produces less drowsiness than does phenobarbital, but the evidence for such claims cannot withstand critical scrutiny.

Thirty years ago Nirvanol was generally considered too dangerous to be useful in epilepsy, and in a recent evaluation of antiepileptic drugs,[14] Nirvanol is tabulated as being too toxic for clinical use. It has been stated[15] that Mesantoin produces rashes in a much lower proportion of patients than does Nirvanol. This conclusion is not supported by the literature. The reputation of Nirvanol for toxicity stems from its use in chorea in children on a dosage schedule that resulted in rashes in almost all patients. In adults receiving doses such as would be used in epilepsy, the incidence of rashes was much lower. There has been no direct comparison of Mesantoin and Nirvanol in the same type of patient and in the same dosage. For the most part the reactions that have occurred with Mesantoin are reminiscent of the earlier reports of Nirvanol toxicity. It is difficult to believe that Nirvanol produced in the body from Mesantoin should be any less likely to evoke a hypersensitivity reaction than the same amount of Nirvanol administered as such.

One patient receiving DMO has experienced photophobia of the same type that occurred when he was taking trimethadione several years before. This characteristic toxic reaction to trimethadione may then be produced entirely or in large part by the metabolic product. No other toxic reactions have as yet been observed in the small number of patients who have received DMO.

From the evidence now available, it appears dubious whether anything is accomplished therapeutically by administration of one of these methylated drugs that cannot be accomplished by administration of the non-methylated compound into which it is converted in the body. Neither is there convincing evidence that the use of the methylated drugs permits a lower incidence of toxic reactions. Whether these methylated drugs offer anything more in practical therapeutics than a devious and expensive means of administering the corresponding non-methylated compounds can well be questioned.

SUMMARY

Through metabolic demethylation mephobarbital (Mebaral) is converted to phenobarbital, metharbital (Gemonil) to barbital, Mesantoin to Nirvanol, trimethadione (Tridione) to 5,5-dimethyl-2,4-oxazolidinedione (DMO), and paramethadione (Paradione) to 5-ethyl-5-methyl-2,4-oxazolidinedione (EMO). These metabolic conversions are almost complete in man. When the methylated drugs are administered in the chronic schedules used clinically, the products of demethylation accumulate in the body in amounts much greater than those of the original drugs. The roles of the demethylated products in therapeutic and toxic effects are discussed, and the question is raised whether for practical purposes the clinical use of one of these methylated drugs amounts only to a devious means of administering the corresponding non-methylated compound.

The authors are indebted to the staffs of the North Carolina State Hospitals at Butner and Raleigh for their cooperation in making patients available for these studies.

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Relation between excitability and metabolism in brain as elucidated by anticonvulsant drugs

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THE SERIES of experiments reported here was designed to evaluate the *in vivo* effects of two anticonvulsant drugs, acetazoleamide (Diamox) and diphenylhydantoin (Dilantin), on electrical excitability, electrolyte and acid-base status, and amino acid metabolism of the cerebral cortex of rats.

A summary of the effects of these two drugs on various experimentally-induced seizures in rats is presented in table 1. Both agents elevate electroshock seizure threshold to the same extent. Both drugs protect against maximally induced seizures in extremely low doses, although acetazoleamide is slightly more potent. However, against the clonic seizures induced by abrupt withdrawal of rats from 35 to 50 per cent concentrations of carbon dioxide, there is a marked difference between the two drugs. This difference gives some insight into the mechanisms of their anticonvulsant action. Diphenylhydantoin protects against withdrawal seizures only in toxic doses, and lower doses actually enhance such seizures. In sharp contrast, acetazoleamide protects against carbon dioxide-induced withdrawal seizures in low doses. Previous studies[1] have indicated that withdrawal seizures are due to a rapid influx of sodium into brain cells, concurrent with the rapid loss of carbon dioxide from the brain. Therefore, one might anticipate that any procedure or drug which prevents such seizures might act by slowing the rate at which sodium enters the cells, thus preventing the rapid depolarization which induces seizures. Acetazoleamide is such a drug. On the other hand, a compound such as diphenylhydantoin, which enhances seizures under these conditions, might also enhance the rate of entrance of sodium into the cells. The following data offer additional evidence for this concept of the action of anticonvulsant drugs.

The effects of acetazoleamide and diphenylhydantoin on electrolyte and acid-base metabolism of the brain are depicted in figure 1. Both drugs markedly decreased intracellular sodium concentration[2,3] (based on chloride space as a measure of extracellular fluid space of brain), and thereby increased the ratio of extracellular to intracellular sodium concentration. Although diphenylhydantoin did not significantly change the ratio of intracellular to extracellular potassium in brain, acetazoleamide significantly increased this ratio. Diphenylhydantoin decreased cellular total carbon dioxide concentration, calculated as a decreased intracellular bicarbonate concentration and pH. Acetazoleamide increased brain cellular total carbon dioxide concentration. Since the inhibition of carbonic anhydrase by acetazoleamide slows the approach to equilibrium of the hydration reaction of carbon dioxide, the calculation of pH by the Henderson-Hasselbach equation (which assumes the presence of equilibrium conditions) may not be valid. Hence the observed increase in cellular total carbon dioxide may then actually indicate a local increase in carbon dioxide concentration, which probably accounts for the anticonvulsant effects of acetazoleamide.[3]

Since both diphenylhydantoin and acetazoleamide decrease intracellular sodium concentration, it was of interest to test the effects of these agents on the uptake of radiosodium (Na^{22}) by brain cells. The results are shown in figure 2. The ordinate is the relative specific activity (tissue S.A./plasma S.A.) and the abscissa is time in hours. Diphenylhydantoin increased the uptake of radiosodium,[2] where-

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TABLE 1

EFFECT OF DIPHENYLHYDANTOIN AND ACETAZOLEAMIDE ON ELECTRICALLY-INDUCED SEIZURES AND ON CO_2-INDUCED WITHDRAWAL SEIZURES.*

Treatment	*Maximal Electroshock Seizures* ED_{50} *mg./kg.*	*Electroshock Seizure Threshold % Increase* Acute	Chronic	*CO_2-Withdrawal Seizures* ED_{50} *mg./kg.*
Diphenylhydantoin	9.0 (7.4–10.0)	12	18	> 150
Acetazoleamide	2.0 (1.6–2.4)	11	14	10

*The values in parentheses are 95 per cent confidence limits.

as acetazoleamide decreased such uptake,[3] as compared with the controls. The acetazoleamide-treated rats were nephrectomized in order to avoid the effects of this drug on the kidney; hence the relative specific activity is lower than that in the diphenylhydantoin-treated group.

The fact that the increased turnover of radiosodium induced by diphenylhydantoin is accompanied by a decreased intracellular sodium concentration can be interpreted to mean that this agent enhances the active extrusion of sodium from brain cells, and thereby lowers intracellular brain sodium concentration. In contrast, the decreased turnover of radiosodium and decreased intracellular sodium concentration induced by acetazoleamide can be interpreted to mean that this agent decreases the influx of sodium into brain cells and thereby decreases intracellular sodium concentration. This decrease in sodium concentration in cells is probably the basis for the general anticonvulsant action of these agents. Thus, the effect of acetazoleamide to protect against carbon dioxide-induced withdrawal seizures can be explained by its ability to decrease permeability to sodium, and the ability of diphenylhydantoin to enhance such seizures can be explained by the increased turnover of sodium produced by this agent.

Some recent studies[4–6] have implicated the glutamic acid-glutamine and aspartic acid-asparagine systems in electrolyte transport in brain tissue. Therefore, the effects of diphenylhydantoin and acetazoleamide on concentrations of free amino acids in brain were determined. The results are depicted in figure 3. Acetazoleamide produced a non-specific increase in the concentrations of most of the amino acids studied. Glutamic and aspartic acid concentrations were increased, as were also glutamine and asparagine concentrations. The changes in free amino acids seem to be related to the accumulation of carbon dioxide produced by the drug. In contrast, however, diphenylhydantoin decreased glutamic and aspartic acid concentrations and increased glutamine and asparagine concentrations. The concentrations of other amino acids were unchanged with the exceptions of histidine, arginine, and lysine which were decreased, and gamma-aminobutyric acid which was increased. Inasmuch as the changes in amino acids induced by diphenylhydantoin seem to be specifically related to changes in the glutamic acid-glutamine and aspartic acid-asparagine systems, the active transport of sodium may be closely coupled to these systems. The effect of acetazoleamide to inhibit sodium influx into brain cells seems to be unrelated to amino acid changes, but to involve the enzyme carbonic anhydrase.

Finally, the effects of diphenylhydantoin and acetazoleamide on brain sodium and potassium concentrations have been correlated with changes in the anion content in brain cells. The results are summarized in table 2. Two sets of controls are tabulated and the differences ($\triangle$) of the drug-treated rats from the appropriate controls are shown. Acetazoleamide increased the sum of sodium and potassium concentrations by 5.2 mM/kg. of cellular water. This increase was accounted for by the slight increase in bicarbonate and excess amino acid anion concentrations. Thus, 4.2/5.2 or 81 per cent of the increase in cation induced by acetazoleamide corresponded to the increase in these cellular anions. In rats made tolerant to the anticonvulsant effects of acetazoleamide, the sum of sodium and potassium concentrations increased by 31.9 mM/kg. cellular water, especially because of

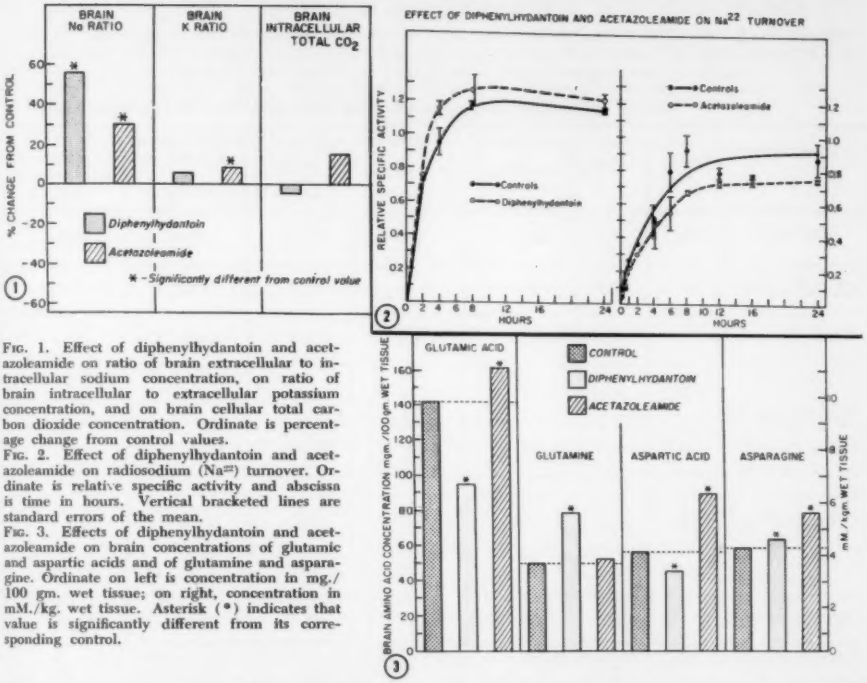

FIG. 1. Effect of diphenylhydantoin and acetazoleamide on ratio of brain extracellular to intracellular sodium concentration, on ratio of brain intracellular to extracellular potassium concentration, and on brain cellular total carbon dioxide concentration. Ordinate is percentage change from control values.

FIG. 2. Effect of diphenylhydantoin and acetazoleamide on radiosodium (Na^{22}) turnover. Ordinate is relative specific activity and abscissa is time in hours. Vertical bracketed lines are standard errors of the mean.

FIG. 3. Effects of diphenylhydantoin and acetazoleamide on brain concentrations of glutamic and aspartic acids and of glutamine and asparagine. Ordinate on left is concentration in mg./100 gm. wet tissue; on right, concentration in mM./kg. wet tissue. Asterisk (*) indicates that value is significantly different from its corresponding control.

the marked increase in cellular potassium concentration. In these animals, one-half of this increase in cation or 15.3 mM corresponded to the increase in amino acid anion. Cellular bicarbonate concentration remained unchanged and hence the remainder of the increment in anion presumably corresponded to an increase in protein anion. It is possible that this increase in protein is due to increased synthesis of carbonic anhydrase as tolerance develops. Diphenylhydantoin decreased the sum of sodium and potassium concentrations in brain cells, because of the reduction in intracellular sodium concentration. This decrease in cation was accompanied by a decrease in intracellular bicarbonate concentration and a net decrease in excess amino acid anion concentration. The decrease in amino acid anion accounted for 3.7/4.0 or 93 per cent of the total cation decrease. Thus, it is apparent that changes in total cation concentration in brain cells are controlled, to a large extent, by changes in free amino acid concentrations of the brain cells. The concentration of potassium in brain cells seems to be closely related to the pH of brain cells. For example, in diphenylhydantoin-treated rats, there is an increase in hydrogen ion concentration (decreased pH) with no change in potassium concentration in brain cells, despite the fact that sodium concentration is decreased. The constancy of cellular potassium, as cellular sodium decreases, can be explained by the fact that total anion is decreased. One might predict that the loss of sodium and potassium would be in proportion to their concentrations in brain cells and, since potassium is present in higher concentration in cells, it should be lost to a greater extent. Inasmuch as diphenylhydantoin also activates the sodium pump, the entire decrease in cellular cation is accounted for by the increase in sodium efflux. The constancy of potassium results from the algebraic summation of two opposing tendencies, the metabolic acidosis and concomitant decrease in anion which tend to decrease total cation concentration and the active extrusion of sodium from brain cells which tends to increase

TABLE 2

COMPARISON OF THE EFFECTS OF DIPHENYLHYDANTOIN AND ACETAZOLEAMIDE IN RATS MADE TOLERANT TO ACETAZOLEAMIDE, ON THE SUM OF SODIUM AND POTASSIUM CONCENTRATIONS IN BRAIN CELLS, AND ON HCO_3 AND EXCESS FREE AMINO ACID ANION CONCENTRATIONS IN BRAIN CELLS*

BRAIN INTRACELLULAR CONCENTRATION mM/Kg. CELLULAR WATER

TREATMENT	Na+K	HCO_3^-	[GLUTAMIC + ASPARTIC ACIDS]	− [ARGININE + HISTIDINE + LYSINE]	= [EXCESS AMINO ACID ANION]	EXCESS AMINO ACID ANION + HCO_3^-	[Na + K] − [EXCESS AMINO ACID ANION + HCO_3^-]
CONTROL I	198.3	15.3	22.7	5.4	17.3	32.6	165.7
ACETAZOLEAMIDE	203.5	16.6	29.4	9.2	20.2	36.8	166.7
Δ FROM CONTROL I	+5.2	+1.3	+6.7	+3.8	+2.9	+4.2	+1.0
TOLERANT TO ACETAZOLEAMIDE	230.2	16.0	38.1	5.5	32.6	48.6	181.6
Δ FROM CONTROL I	+31.9	+0.7	+15.4	+0.1	+15.3	+16.0	+15.9
CONTROL II	193.7	13.5	24.8	10.5	14.3	27.8	165.9
DIPHENYL-HYDANTOIN	189.7	12.8	18.1	7.5	10.6	23.4	166.3
Δ FROM CONTROL II	−4.0	−0.7	−6.7	−3.0	−3.7	−4.4	+0.4

*Calculations of intracellular HCO_3 concentration were based on the method of Wallace and Hastings.[8]

potassium concentration.[7] Acetazoleamide, in contrast, increases amino acid anion to a greater extent than it decreases intracellular sodium concentration and, as a result, the drug increases potassium concentration in brain cells.

Potassium concentration in brain cells seems to be regulated by two factors: 1) the total anion content of the cell, which may vary considerably and is dependent on the cellular pH as well as on the metabolic processes which affect the concentrations of free acidic and basic amino acids and on the concentration of bicarbonate in brain cells, and 2) the coupling of potassium movement to active sodium transport. On the other hand, sodium concentration in brain cells seems to be regulated by the balance between an active extrusion process and passive influx and is only slightly affected by total anion content of the cell. Intracellular brain sodium concentration can, thus, be influenced by anticonvulsant agents which decrease intracellular sodium concentration by enhancing the active sodium extrusion process, such as diphenylhydantoin, or which decrease intracellular sodium concentration by decreasing sodium influx, such as acetazoleamide.

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Sound-induced seizures in animals

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SOUND-INDUCED SEIZURES in animals have been observed and reported from time to time,[1,2] but only recently have they been studied extensively and methods devised to produce them consistantly in a large percentage of a group of animals. Despite the fact that approximately 250 reports concerning sound-induced seizures have appeared during the past 20 years, the phenomenon has attracted relatively little attention from physicians and medical investigators, perhaps because most of the reported studies have appeared in psychologic journals. The research possibilities of sound-induced seizures in relation to seizure states in man have been further obscured because many reports have been concerned with the attacks as a supposed abnormal or neurotic form of behavior,[3] and another group of reports has considered them primarily as incidental to dietary deficiencies of certain substances such as magnesium.[4] Fortunately, for anyone desiring a familiarity with the subject, two excellent reviews have appeared, one by Finger[5] covering the literature up to 1947 and one by Bevan[6] covering the literature from 1947 to 1954.

With the methods presently available, convulsive seizures can be produced consistantly in 80 to 100 per cent of a group of animals. The procedures for accomplishing this are relatively simple. In our studies we have used two methods: 1) selection of a strain of animals with a high degree of susceptibility to sound-induced seizures on an hereditary basis, and 2) production of sensitivity to sound-induced seizures by creating a magnesium deficiency.

HEREDITY AND SOUND-INDUCED SEIZURES

A number of investigators have observed that strains of rats vary in their sensitivity to sound-induced seizures, some strains showing no convulsions and some strains showing up to 50 or 60 per cent response.[7,8] As yet no one has developed and maintained a strain of rats bred for this purpose alone, but certain recognized strains are rather high in seizure response. Recently, in testing various strains of rats, investigators at the National Institutes of Health have found about 50 per cent susceptibility in an albino strain M-520.

Strains of mice with higher susceptibility have been found[9] and one of these, the DBA/2 strain, shows 90 to 100 per cent response. This is a highly inbred strain of mice which has been carried through well over 200 generations of brother-sister mating beginning in 1909. This strain is now maintained at the Roscoe B. Jackson Memorial Laboratory in Bar Harbor, Maine and is available for investigators. In this strain occurence of seizures varies with age, beginning around 20 days and reaching a peak at 30 days. Vicari[10] has studied this variation in response with age in 5,297 mice and the results are shown in figure 1. In DBA/2 mice shipped from Bar Harbor to our laboratory in Charlottesville, Virginia, seizures can be consistantly reproduced and we have seen little difference in males and females. A summary of seizures upon first stimulation in 428 mice used as controls in our laboratory experiments during 1955 is shown in Table 1. Not all of these animals were tested during their period of highest response.

In mice of the DBA/2 strain the seizures often result in death, apparently from respiratory paralysis. This is probably a separate genetically determined characteristic but is useful as an additional method of grading the effects of drugs or other agents. This characteristic limits the usefulness of this strain for any experiment where repeated tests are necessary. Rats seldom die after sound-induced seizures and, therefore, the same animal can be used repeatedly. At present there is a definite need for a strain of rats with as high a susceptibility to sound-induced seizures as the DBA/2 strain of mice. Development of such a strain of rats would make both chemical and electrical study of brain activity easier because of the larger size of the animal. Also, studies of re-

From the department of neurology and psychiatry, University of Virginia School of Medicine, Charlottesville, Virginia.
Read in abbreviated form at the first annual meeting of the Section on Neurochemistry, American Academy of Neurology, Boston, April 27, 1957.
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TABLE 1
INCIDENCE OF SEIZURES IN 428 DBA/2 MICE USED AS CONTROLS

Total	Sex	No. of Seizures	% Seizures	% Deaths
428	M&F	345	80.6	31.1
215	M	177	82.3	30.7
213	F	168	78.9	31.5

peated convulsions could be made since it is unlikely that seizures would be fatal in the rats.

MAGNESIUM DEFICIENCY AND SOUND-INDUCED SEIZURES

The observation that magnesium deficiency produces convulsions in various animals has been made by a number of investigators.[11,12] Seizures occur spontaneously if the deficiency is severe enough but before this point is reached the animals show a greatly increased sensitivity to induction of seizures by sound stimulation. In our studies we have produced magnesium deficiency in rats for the dual purpose of studying the effects of electrolyte changes on brain function and of assessing the efficacy of anticonvulsant drugs. Magnesium deficiency can be produced rather easily if the restriction of magnesium is begun during the period of growth, when it appears the demands for magnesium are increased. Production of a high degree of susceptibility to sound-induced seizures in rats on a magnesium deficient diet is demonstrated in figure 2.

It is possible that a species difference and also a strain difference in susceptibility to magnesium deficiency exist. In our laboratory we have been able to produce the syndrome in both albinos and hooded rats. Also, DBA/2 mice put on a magnesium deficient diet after they have become older and lost their natural susceptibility to sound-induced seizures once again become susceptible to sound-induced seizures. Others have reported producing seizures in golden hamsters by magnesium deficiency.[13]

METHODS

Diet. In our studies we have used a diet essentially like that of Kruse, Orent, and McCollum.[4] This diet contains approximately 1.0 mg. magnesium per 100 gm.

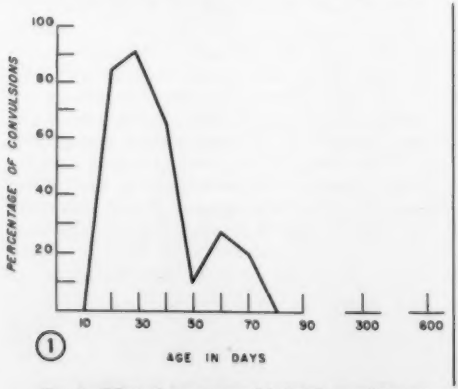
Ingredients:

*Starch	560 grams
Casein (purified)	200 grams
Calcium carbonate	15 grams
Potassium chloride	10 grams
Sodium bicarbonate	7 grams
Ferric ammonium sulfate	5 grams
Sodium chloride	5 grams
Viosterol	15 drops
Nucoa	80 grams
Potassium phosphate	17 grams
**Alcohol (extract of yeast	100 ml.

*The starch (Argo) must first be made magnesium-free. This is done by adding 2,000 ml. of 5 per cent HCl to 1 pound of starch. (To prepare 5 per cent HCl add 88 ml. of concentrated HCl to 1,912 ml. of distilled water.) Mix with starch. After the starch has settled, the supernatant is drawn off and the starch is washed with distilled water. The washing process is repeated until the starch has been washed three times with distilled water. The starch is then poured onto trays placed in front of a fan or air conditioner and allowed to dry.

**To prepare the alcoholic extract of yeast, weigh out 150 gm. of powdered yeast and add 825 ml. of 95 per cent alcohol and 675 ml. of distilled water. Mix and allow yeast to settle. Pour off supernatant extract for use in diet.

The diet is poured onto trays and allowed to dry in front of a fan or air conditioner in the same manner as the starch.



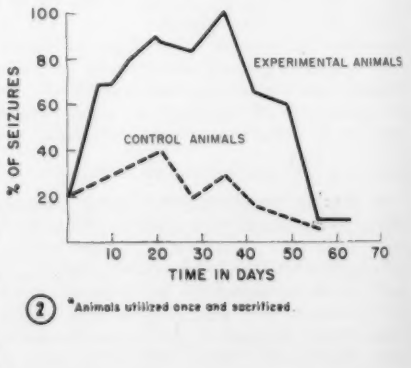


FIG. 1. Effect of age on sound-induced convulsive seizures.
FIG. 2. Seizure susceptibility in male albino rats on a magnesium deficient diet. Total number of subjects—170.

Apparatus. The apparatus used for sound stimulation is similar to that described by other investigators.[6] An electric doorbell is suspended in the center of a galvanized tub 15 inches in diameter and 8 inches high and the tub set in sawdust for sound insulation in a plywood box (24 by 24 by 24 inches) with an observation window in the top. The animals are allowed complete freedom of movement within the tub. Stimulation consisted of ringing the bell for 60 seconds, the approximate sound level produced being 90 to 92 decibels.

SEIZURE PATTERN

The attacks are very similar in mice and rats and consist essentially of a period of wild and rapid running around the enclosure, followed by a tonic-clonic convulsion and then a period of recovery. Some animals run wildly but never convulse and this has been considered part of the seizure by some observers. Animals protected by anticonvulsants do not show this running activity. At times there will be two periods of running, with the seizure following the second one. During the period of recovery the animal is usually in apparent coma for a short period and gradually returns to normal awareness and activity. During the period of recovery, *flexibilitas cerea* as well as attacks of furor have been observed. Death results in many of the mice, though occasionally this can be prevented by immediate artifical respiration.

The running prior to the convulsion is such a common feature that many have considered it as a necessary prelude to the seizure and have reported that restraint of the animal to prevent running will prevent the convulsion.[14] This is a factor that needs further study, since it is difficult to understand how running could cause the attacks. We have made no large study of this factor, but we have produced convulsions followed by death in DBA/2 mice, despite the fact they were completely restrained by binding the legs and body with adhesive tape.

The tonic-clonic convulsion is similar in all respects to that produced by electroshock or Metrazol and would seem to be a counterpart of a generalized convulsion or so-called grand mal seizure in man. Despite the large number of reports on sound-induced seizures, very few investigators have attempted to record EEG's on the animals either between or during attacks, and these investigators differ as to whether a seizure discharge was recorded or whether the changes are simply movement and muscle artifact.[15,16] This aspect of the sound-induced seizures certainly needs further study.

CHARACTERISTICS OF THE STIMULI

All observers agree that mixed noise, such as that produced by a bell, jingling keys, or an air blast, is more effective in inducing seizures than any pure tones. Tones in the 8,000 to 11,000 cps range appear to be the most effective, but in some animals frequencies up to 35 kc. have resulted in seizures.[6] Theoretically a mixed noise should stimulate more nerve fibers than a pure tone and thus lead to a greater total inflow of stimulus. There have been reports that a pure tone is more effective if it is interrupted. This suggests some similarity to the characteristics of photic stimulation in man, which is more effective as a flicker and perhaps more effective as total area of retina stimulated is increased. It would seem possible that sound in animals such as mice and rats and flickering light in humans both act to provoke convulsions by excitation of a so-called centrencephalic system. It is interesting that hereditary forms of photogenic epilepsy in man[17] have been observed, just as audiogenic seizures can be hereditary in animals.

COMPARISON WITH EPILEPSY IN MAN

There is always danger in carrying conclusions from the experimental animal to man. Nevertheless, sound-induced seizures in animals can be hereditary. They show a marked change with age, reaching a peak at "adolescence" in the animal, and there is no evidence of any anatomic or structural basis for the attacks. Studies carried out in our laboratory[18] (to be reported separately) show that animals are protected from sound-induced seizures by the same drugs which are of proved value in control of epilepsy in man, and they are not controlled by other drugs which have not been effective in man. Thus in many ways sound-induced seizures in animals would seem to be comparable to the hereditary type of so-called centrencephalic seizures in man. If this is true, then animals susceptible to sound-induced seizures constitute an important area of investigation, offering both a means of testing drugs for anticonvulsant properties and also a means of investigating the chemical and metabolic processes involved.[19,20]

SUMMARY

The characteristics of sound-induced seizures in animals are described and methods for producing them consistantly are outlined.

Sound-induced seizures in animals resemble epilepsy in man in some respects, which offer an area of investigation that may reveal factors which will be helpful in the understanding of this condition.

Specific suggestions include development of a strain of rats with a high percentage of susceptibility to sound-induced seizures and more study of electroencephalographic changes associated with sound-induced seizures.

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Sound-induced seizures in animals:

The efficiency of certain anticonvulsants in controlling sound-induced seizures in DBA/2 mice

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DISCOVERY and development of the large number of anticonvulsant drugs now available for the treatment of epilepsy has been accomplished primarily by empirical testing of drugs for anticonvulsant properties in experimental animals. A variety of procedures and animals have been used for this purpose,[1–3] but certainly no one procedure can be expected to test all the anticonvulsant properties that might be of value in epilepsy in man. With the fairly recent introduction of the DBA/2 strain of mice,[4] 80 to 100 per cent of which are susceptible to sound-induced seizures, another assay method for evaluating the anticonvulsant properties of drugs has become available.

We have carried out a series of studies to demonstrate the usefulness of DBA/2 strain of mice as an assay method for anticonvulsant drugs, and at the same time to determine the dimensions of action of three common anticonvulsants to serve as a baseline for evaluation of other drugs supposed to have similar properties. The drugs studied were sodium phenobarbital, diphenylhydantoin sodium (Dilantin), and trimethadione (Tridione); the study was designed to find for each drug the lowest dose giving 100 per cent protection from sound-induced seizures, the lowest dose preventing lethal seizures, the length of time the lowest effective dose gave protection, and the dose at which clinical signs of toxicity developed.

METHODS

The DBA/2 mice used in this study were purchased from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, and were between ages 28 to 35 days when tested. Approximately equal numbers of males and females were used, the data being analyzed separately for each sex; since no significant difference occurred in seizure frequency, the data are reported without sex differentiation. The average weight of the mice was 13.2 gm. All tests were carried out in the stimulus box which we have previously described[5] and test stimuli given for 60 seconds. All tests constituted the first exposure to the auditory stimulus unless otherwise noted. No animal was considered to have had a seizure unless a generalized tonic-clonic convulsion occurred.

All medications were given intraperitoneally with a No. 25 needle. Sodium phenobarbital and diphenylhydantoin sodium were dissolved in distilled water and a commercial solution of trimethadione was used. Size of injections varied from 0.1 to 0.4 cc. All dosage was calculated in mg./kg., each group of mice being weighed separately and the average weight used for dosage calculation.

To rule out the possibility that the trauma of injection alone might change seizure susceptibility, 36 mice were injected with 0.4 cc. normal saline, 35 mice had a dry needle introduced into the peritoneal space, and 39 had no injection. These groups were then tested for seizures with the following results:

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	Number Rats	Per Cent Seizures
Saline	36	80.6
Dry needle	35	80.0
No injection	39	87.2
Total	110	82.7

Calculation shows $X^2 = .8418$ which yields with 2 df, a $P = 0.5$. Hence injection alone was not considered a significant factor.

SODIUM PHENOBARBITAL

A total of 238 mice were divided into eight groups as follows:

	Dosage	Number of Mice
Group A	130 mg./kg.	25
Group B	65 mg./kg.	25
Group C	32.5 mg./kg.	26
Group D	16.25 mg./kg.	25
Group E	8.13 mg./kg.	44
Group F	4.06 mg./kg.	43
Group G	2.03 mg./kg.	25
Group H	Control	25

From figure 1 it is apparent that a dosage of 8.13 mg./kg. produced significant seizure protection and 16.25 mg./kg. produced 100 per cent protection. Thus, it is assumed that the lowest 100 per cent effective dose lies between 8 and 16 mg./kg. The dosage of 4.06 mg./kg. gave only slight seizure protection, but no lethal seizures occurred. No effect could be demonstrated for 2.06 mg./kg. Three observers evaluated signs of toxicity independently and agreed that those receiving 130 mg./kg. were clearly toxic, all mice showing ataxia, lethargy, ruffled pelts, and dulled eyes. The most marked level of toxicity appeared at one hour following injection, and this was used as the optimal period between injection and stimulus for the other groups. The group receiving 32.5 mg. showed only slight reduction in in-cage activity, without other signs of toxicity. No toxic effects could be noted in the smaller dosages.

The minimal protective dosage of 16.25 mg./kg. was then given to four groups each composed of 20 mice. One group was subjected to stimulus at two hours, another at four, the third at eight, and the fourth at 24 hours, with the results shown in figure 2. Separate groups were used in order to negate any effect on seizure incidence produced by repeated stimulus. From these data it would appear that phenobarbital at a dose of 16.25 mg./kg. affords complete protection through eight hours and that some protection against lethal seizures still exists 24 hours post-injection. (The single seizure occurring at two hours in group 1 was ignored.)

DILANTIN (DIPHENYLHYDANTOIN SODIUM)

A total of 200 mice were assigned randomly into eight equal groups. Dilantin was dissolved in freshly boiled distilled water with two or three drops of sodium hydroxide added to increase solubility. As the signs of toxicity at higher dosages appeared maximal at approximately one hour, this was used as the interval between injection and stimulus. The groups were divided as follows:

	Dosage	Number of Mice
*Group A	75 mg./kg.	25
*Group B	66.7 mg./kg.	25
Group C	50 mg./kg.	25
Group D	33 mg./kg.	25
Group E	25 mg./kg.	25
Group F	16.7 mg./kg.	25
Group G	8.3 mg./kg.	25
Group H	Control	25

*The animals in groups A and B had previously been used as controls in the phenobarbital study, but were re-used to determine toxicity since lower doses had produced 100 per cent seizure protection.

Figure 3 illustrates that the lowest 100 per cent seizure protection dosage is between 25 and 33 mg./kg. No seizure protection was obtained at 8.3 mg./kg. but the animals were protected from lethal seizures. No toxic effects were noted under dosages of 75 mg./kg., but animals at this dosage exhibited varying degrees of ataxia and lethargy. Although one animal receiving 25 mg./kg. had a seizure, this dosage was chosen as being closer to the lowest 100 per cent effective dosage than 33 mg./kg. for purposes of determining length of protection. Figure 4 illustrates the results. These results are based on four groups of animals, 25 in each group, tested once at four, eight, 12 and 24 hours, respectively. Thus, significant protection was afforded through eight hours post-injection, and no appreciable seizure protection was demonstrable at 24 hours. The percentage of lethal seizures is still reduced 24 hours after injection.

TRIDIONE (TRIMETHADIONE)

A total of 220 mice were placed randomly in seven groups. From 0.1 to 0.4 cc. of a commercially available Tridione suspension was injected intraperitoneally in the experimental groups.

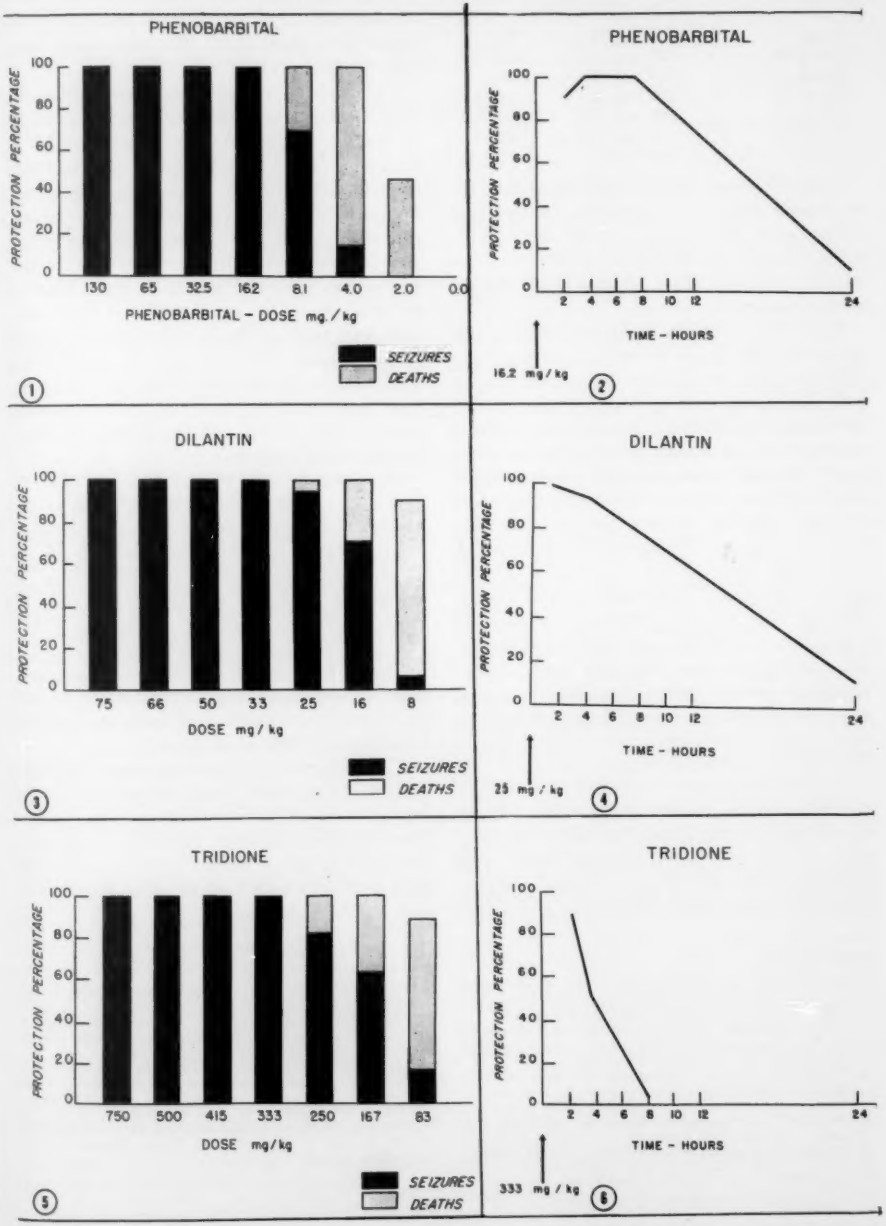

FIG. 1. Protective effects of various doses of phenobarbital on sound-induced seizures. Solid bars denote protection against seizures. Light bars denote no seizure protection but protection against death. FIG. 2. Duration of effectiveness of a single protective dose of phenobarbital against sound stimuli applied up to 24 hours after the dose. FIG. 3. Data for Dilantin in experiments analogous to figure 1. FIG. 4. Data for Dilantin in experiments analogous to figure 2. FIG. 5. Data for Tridione in experiments analogous to figures 1 and 3. FIG. 6. Data for Tridione in experiments analogous to figures 2 and 4.

	Dosage	Number of Mice
Group A	500 mg./kg.	24
Group B	415 mg./kg.	24
Group C	333 mg./kg.	25
Group D	250 mg./kg.	26
Group E	167 mg./kg.	26
Group F	83 mg./kg.	25
Group G	Control	70

Figure 5 shows that 333 mg./kg. is the lowest dosage tested that produced 100 per cent seizure protection, but one-half this dosage still produced very significant protection from seizures and 100 per cent protection against lethal seizures.

Toxic effects were not observed until a dosage of 500 mg./kg. was reached. Doses up to 1,000 mg./kg. were given to smaller groups with increasing lethargy and ataxia, and at 1,000 mg./kg. three of ten animals expired. Again maximum toxicity appeared at one hour, so this was the interval chosen for stimulation after injection. Figure 6 illustrates the effect at longer intervals in a new group of 100 mice given 333 mg./kg. Groups of 25 subjects each were tested once at two, four, eight, and 24 hours, respectively. Evidently at this dosage 100 per cent seizure protection is afforded only under two hours, with significant protection lasting between four and eight hours. Significant protection against lethal seizures was evident at eight hours but not at 24.

DISCUSSION

Typical generalized tonic-clonic convulsions can be produced consistently in 80 to 100 per cent of mice of the DBA/2 strain by the ringing of a door-bell in an enclosed area. This provides a very simple and convenient means of testing anticonvulsant properties and allows a relatively large number of animals to be tested in a short time, thus increasing reliability of the results.

The studies reported above demonstrate the extreme consistency of results in the controls, as well as the sensitivity of this method in measuring the anticonvulsant potency and length of action of the known anticonvulsants studied. Other studies now in progress suggest that the degree of anticonvulsant action seen in tests on the DBA/2 strain of mice correlates very well with effects in man. Most of the animals in the study reported here were used only once so there could be no question of the results. However, in other screening tests we have been able to use the same animals several times, particularly when the substance being tested gave protection at least against lethal seizure.

The doses of anticonvulsants found effective in these animal studies, while considerably higher than the per weight dose used in man, compare well with effective dose levels of these same substances as tested by other experimental means in animals.[6–8]

CONCLUSION

The DBA/2 strain of mice with susceptibility to sound-induced seizures offers a simple yet accurate method of assay for anticonvulsant properties of drugs.

The properties of three common anticonvulsant drugs were clearly defined by testing for effect against sound-induced seizures in DBA/2 mice. Lowest effective dose, toxic dose, and length of action were found to compare well with drug tests by other experiments and clinical experience.

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Sound-induced seizures in animals:

Magnesium deficiency and sound-induced seizures in rats

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BEGINNING with the studies of Kruse, Orent, and McCollum[1] in 1932, there have been many reports of convulsions associated with magnesium deficiency in animals.[2-6] We have studied magnesium deficiency in rats both as a means of producing seizure-susceptible animals and for the purpose of measuring certain associated chemical changes, especially changes in electrolytes. Though we have been able to produce the syndrome of magnesium deficiency with susceptibility to sound-induced seizures consistently, the chemical changes measured do not adequately explain the change in seizure threshold. Nevertheless, there have been certain consistent findings that seem worthy of reporting at this time.

METHODS

The magnesium deficient diet used and also the apparatus for producing seizures by stimulation with sound have been described previously.[7] The blood and tissue specimens were obtained as follows: Immediately after exposure to sound, animals were sacrificed by decapitation and the blood was collected in a 10 cc. tube. Autopsies were performed and tissues placed in crucibles which were subsequently dried and weighed. The following methods were utilized for chemical assay of the various specimens.

Magnesium: Microdetermination with Coleman Model 14 spectrophotometer.[8]

Calcium: Photoelectric determination of oxalic acid and calcium.[9]

Sodium and potassium: Determinations made utilizing the Beckman DU Flame photometer.

INCREASED SUSCEPTIBILITY TO SOUND-INDUCED SEIZURES AND MAGNESIUM DEFICIENCY

The increased susceptibility to sound-induced seizures brought about by magnesium deficiency was tested in two groups of animals. The first group consisted of 100 rats divided into 50 controls and 50 experimentals, which were further divided into four groups. Groups 1 and 2 were placed on the experimental diet and groups 3 and 4, designated as controls, continued on the ordinary laboratory diet. These animals were tested for susceptibility to sound-induced seizures twice weekly. However, one group of experimentals and one group of controls were tested twice weekly, beginning on the third day of the experimental diet and continuing throughout the experiment. Another group of controls and experimentals were not tested for susceptibility to seizures until the twenty-first day and then were tested twice a week throughout the duration of the experiment. Since the percentage of convulsive episodes to sound in each control and experimental group was approximately the same, they have been combined and the results are illustrated in figure 1.

From this experiment it is seen that increased susceptibility to sound-induced seizures can be produced rapidly and in a large percentage of animals. Since the group first stimulated at 21 days showed little difference from that stimulated twice weekly from the beginning, it would appear that repeated stimulation at the rate of twice a week at least has little effect upon continued susceptibility to seizures. Furthermore, it would appear that such stimulation had little effect on the controls.

The second group of animals tested consisted of 170 animals, and this experiment was designed so that each animal would be tested for susceptibility to sound-induced seizures

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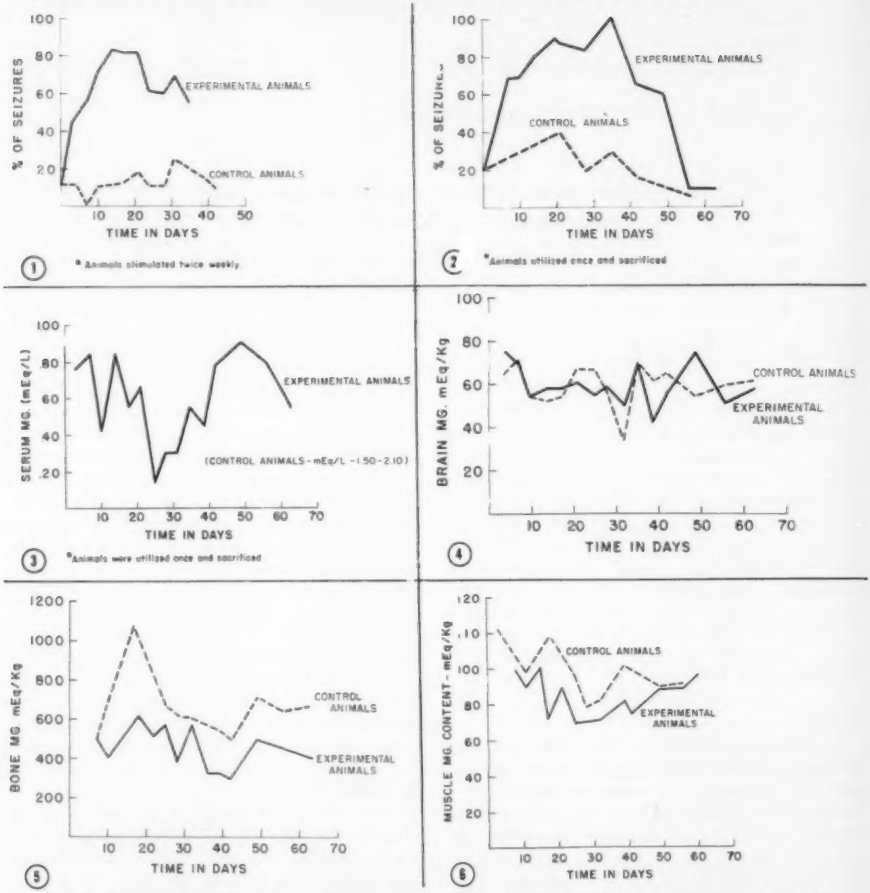


Fig. 1. Seizure susceptibility in male albino rats on a magnesium deficient diet. Total number of subjects – 25 experimental and 50 controls.
Fig. 2. Seizure susceptibility in male albino rats on a magnesium deficient diet. Total number of subjects – 170.
Fig. 3. Blood serum levels of magnesium (in mEq./l) in male albino rats on a magnesium deficient diet. Total number of subjects – 260 experimental and 60 controls.
Fig. 4. Brain magnesium content in mEq./kg. from male albino rats on a magnesium deficient diet.
Fig. 5. Bone magnesium content in mEq./kg. from male albino rats on a magnesium deficient diet.
Fig. 6. Muscle magnesium content from rats on a magnesium deficient diet.

only once. During the experiment animals were sacrificed for tissue determination. These animals were divided into two groups and each week eight or ten animals from both the experimental and control groups were chosen at random, subjected to sound stimulation, and sacrificed. This was continued for nine weeks and results are shown in figure 2. It is evident from this experiment that increased susceptibility to sound-induced seizures reaches a high level, but seems to decrease after a period of time. Since these animals were stimulated only once with sound, this decrease cannot be attributed to the effect of repeated stimulation with sound. Since tissue studies showed an adjustment of magnesium level, even on the magnesium deficient diet, it is possible that the eventual decrease in seizure susceptibility is due to this adjustment of the animal. Another possibility is that age plays a part in suscepti-

bility to seizures and that, with increasing age, susceptibility is less even in the face of magnesium deficiency.

TISSUE LEVELS OF ELECTROLYTES IN MAGNESIUM DEFICIENT ANIMALS

We have followed the serum and tissue levels of magnesium in a number of rats on a magnesium deficient diet. Since the findings that we will report have been consistent, we have combined several groups for the purposes of this report.

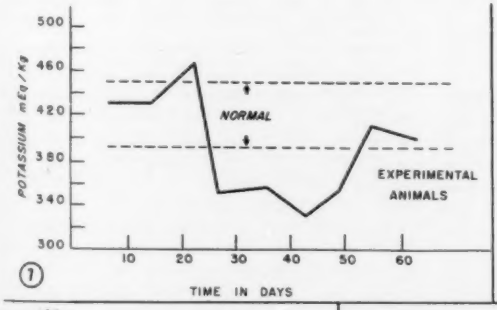
Serum magnesium determinations in a total of 260 experimental animals are shown in figure 3. This shows a variation in degree of magnesium deficiency reaching its greatest degree between 20 and 30 days, with serum magnesium rising again, but never to normal, after 50 or 60 days. We have not carried the test beyound 65 days, though there is some indication that there would once again be a depression. Both from these tests and from clinical observations of the animals as to susceptibility to seizures, general irritability, and vasodilitation of the skin, it appears that the animal definitely makes adjustments to the magnesium deficient diet and apparently has repeated breaks in this adjustment as body stores of magnesium are called upon. The animal, however, apparently holds onto magnesium so that, even in the presence of rather severe deficiency, the animal can adjust for a relatively long time. This is particularly true if the deficiency is not tremendously severe during the period of growth.

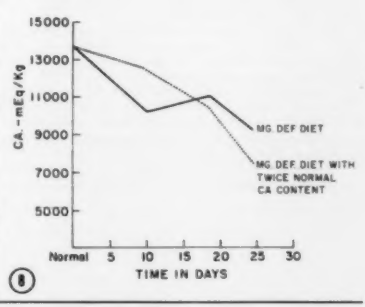
Test of total brain magnesium content in a similar group of experimental animals and controls shows some fluctuation, but no apparently significant depression of tissue magnesium in the experimental animals. This is despite definite severe lowering of serum magnesium. This is demonstrated in figure 4.

This finding that total brain magnesium is not depressed has been consistent. This differs from the work of some investigators who have found depression of magnesium in the brain,[3] but agrees with the studies of Blaxter and Rook[5] of magnesium deficiency in calves.

Levels of bone magnesium determined in a similar group of animals show a depression of bone magnesium. However, this is not great and apparently does not continue to go down or go down as dramatically as the serum levels. This is shown in figure 5.

Magnesium content of muscles in magnesium deficient animals also shows a slight depression





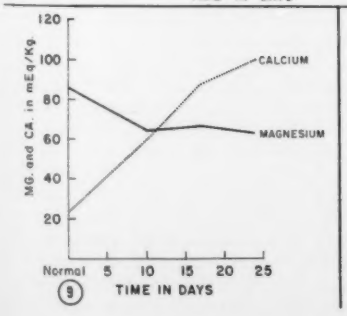


FIG. 7. Muscle potassium from rats on a magnesium deficient diet.

FIG. 8. Calcium content of serum from rats on a magnesium deficient diet. Total number of subjects – 60.

FIG. 9. Magnesium and calcium content of kidneys from rats on a magnesium deficient diet. Total number of subjects – 60.

when compared with controls, but follows more nearly the curve of serum magnesium, going down and then apparently adjusting upward. This is shown in figure 6.

Somewhat similar changes in muscle potassium also occur and this is shown in figure 7. There appears to be a depression of muscle potassium at the time when serum magnesium in the experimental animals shows the greatest depression, and then some adjustment upward occurs as the serum magnesium levels rise.

Calcium determinations were not done on all of our experimental animals, but we do have calcium determinations of serum and kidney calcium levels on a group of 60 animals. In these animals the serum calcium did not change from the control group, even when the magnesium deficient animals were fed a double amount of calcium in their diet (figure 8). There were marked changes of calcium in kidney tissue, however, and this is shown in figure 9. During the time of this experiment while the animals were on a magnesium deficient diet, kidney content of calcium rose steadily, whereas magnesium content stayed approximately the same.

It is interesting to note in this same group of animals that, in those fed an additional amount of calcium, there was no added protection against sound-induced seizures.

CONCLUSIONS

A high percentage of susceptibility to sound-induced seizures can be induced in rats by a magnesium deficient diet.

Serum levels of magnesium tend to show a depression reaching its lowest point between 20 and 30 days and then adjusting upward, but not returning to normal.

The total amount of magnesium in the brain does not appear to change, despite severe lowering of serum magnesium.

With magnesium deficiency there is a marked deposition of calcium in the kidneys.

With magnesium deficiency there appears to be an associated slight lowering of magnesium and potassium in muscle.

Additional calcium given to animals rendered magnesium deficient does not protect them from sound-induced seizures.

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Composition of cerebrospinal fluid under varying conditions

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THERE IS an increasing amount of evidence which suggests that the cerebrospinal fluid is in equilibrium with and probably equivalent to the interstitial phase of brain tissue. Assuming this to be true, the electrolyte concentrations which have low potassium and high sodium in the cerebrospinal fluid suggest that this extracellular phase is being metabolically maintained to provide a favorable environment for the conduction of nervous impulses, whose propagation is associated with the membrane potential resulting from the differences in intra- and extracellular concentrations of potassium and sodium ions. The maintenance of such a special environment could be a function of the glia, which have often been thought to have additional metabolic functions as well as providing the structural framework of the brain.

As a first step in the examination of this hypothesis, the production and composition of cerebrospinal fluid is being examined under a variety of conditions which affect cerebral metabolism. This paper is a preliminary report on the work to date, and is concerned chiefly with methods, establishment of normal conditions, and some critical experiments relative to the basic assumptions.

Studies have been carried out on the flow rate and composition of cerebrospinal fluid from the cisterna magna of normal dogs and from the ventricles of dogs made hydrocephalic by cisternal injections of kaolin. Composition studies include measurement of both serum and cerebrospinal fluid total osmotic pressure, potassium ion concentration, sodium ion concentration, sugar concentration, free and combined neuraminic acid concentration, and cerebrospinal fluid total protein concentration. Experiments have been carried out to determine the effect on rate of flow and composition by variation of the drainage pressure, by extirpation of the choroid plexuses, by changing total serum osmotic pressure, and by the intravenous administration of acetazoleamide (Diamox).[1]

The rate of flow of cerebrospinal fluid in the cisterna magna of a normal dog under Nembutal anesthesia lying on his right side receiving no intravenous fluid showed a linear relationship with the flow of pressure from a point about equal to the venous pressure of the superior longitudinal sinus (about 70 mm. of water) to about −30 mm. of water, the zero point being the level of the right atrium of the heart. Below this flow pressure of −30 mm. down to about −100 mm. of water (the lowest pressure studied), the flow appeared to be relatively constant within the limits of experimental variation.

Flow of cerebrospinal fluid from the ventricles of the hydrocephalic dogs showed a similar linear relationship, but the pressure at which zero flow occurred was somewhat higher. This pressure (about 150 mm. of water) did, however, correspond to the sagittal sinus venous pressure which is elevated in hydrocephalic dogs. Another difference was that the maximum flow rate of cerebrospinal fluid from the hydrocephalic dogs never was as great as from the cisterna magna of the normal dog. The composition of cerebrospinal fluid was unchanged during six hours of continuous drainage, even at the maximum rates. One important feature of the composition studies was the very stable level of potassium concentration. Other constituents showed fluctuations associated with changes in the serum levels, but potassium remained unchanged in spite of serum changes. Neuraminic acid remained constant as long as the protein did not change. If the protein increased, then neuraminic acid values would change correspondingly.

As composition was unaffected by the conditions of maximum flow, other experiments were carried out under these conditions since it was felt that any real changes could be more easily detected.

Changes in total serum osmotic pressure affected both rate of flow and cerebrospinal fluid composition. Increasing the serum total osmotic

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pressure caused a decrease in the cerebrospinal fluid flow rate and an increase of total osmotic pressure, protein and sodium concentration, but the potassium concentration remained unchanged. Decreasing the serum total osmotic pressure caused an increase in cerebrospinal fluid production, with a dilution of protein and sodium, while potassium again remained unchanged. Flow rate changes occurred exponentially with time, which suggests that they were passively related to the water and sodium shifts. The mechanism of the constancy of potassium concentration is not clear.

The choroid plexuses were removed from the lateral ventricles of normal and hydrocephalic dogs. In the hydrocephalic dog the foramen of Monro was plugged, the plexus was removed from only one ventricle or from both lateral ventricles, and the third ventricle was occluded with gel foam. Anatomic studies at autopsy confirmed the completeness of the plexectomies. In most cases they were complete, although in some animals a microscopic tuft of plexus was found in the tip of the temporal horn. In no case did the remaining plexus amount to more than 5 to 10 per cent of the total in the ventricle. Flow studies were made one to four months after surgery. Some 25 experiments have been carried out without finding any difference in either rate of flow or cerebrospinal fluid composition after choroid plexectomy. It is of particular importance that the potassium levels have remained unchanged with the same low ratio compared to serum potassium levels. It is also of some interest to note that neuraminic acid concentration is the same before and after plexectomy, confirming Uzman's conclusion[2] that this substance comes from the brain.

Acetazoleamide (Diamox) has been reported to cause a decrease in cerebrospinal fluid flow, presumably because of its effects upon cerebral metabolism.[1] It has not been possible to confirm this observation. Intravenous Diamox in the amunt of 150 mg./kg. has failed to significantly change cerebrospinal fluid flow, although it does produce a wide variety of other physiologic changes. One factor not controlled in the original experiments was the osmotic pressure of the injected Diamox which was given as a solution of 100 mg./cc. Such a solution has three to four times the osmolarity of serum. Therefore, it would have the effects attendant upon the injection of any hypertonic solution, and would be expected to cause a decrease in cerebrospinal fluid production. Diamox injected as an isotonic solution did affect the composition of the cerebrospinal fluid slightly, since the sodium followed the serum sodium changes and the protein was increased in some experiments, but more data are needed to confirm this. Potassium was unchanged.

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